

Division of Nutritional Epidemiology  
Institute of Environmental Medicine, Karolinska Institutet

# **VITAMIN D AND BLOOD PRESSURE**

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## ABSTRACT

The physiological function of circulating 25-hydroxyvitamin D (25(OH)D) is to maintain serum calcium range that supports skeletal system. Evidence is mounting that vitamin D has beneficial effects on other important functions in tissues not primarily related to mineral metabolism. Circulating 25(OH)D levels are influenced both by the amount of ultraviolet B exposure from the sun to the skin and dietary intake.

The aims of this thesis were to: 1) examine the predictors of serum 25(OH)D levels both during winter and summer among women from the Swedish Mammography Cohort (SMC) in central Sweden (latitude 60° N). 2) evaluate the association between plasma 25(OH)D levels and the prevalence of hypertension among men in the Uppsala Longitudinal Study of Adult Men (ULSAM). 3) evaluate the association between serum vitamin D levels and blood pressure among women in the SMC. 4) quantitatively summarize the evidence from published studies on the association between circulating 25(OH)D and hypertension.

In 116 women (61-86 years), investigated regarding determinants of serum 25(OH)D concentrations during winter, mean concentrations were 69 nmol/L. In a multiple linear regression model serum 25(OH)D concentrations were associated with dietary vitamin D, travel to sunny location during winter and the use of dietary supplements. Among these women 100 participated again in an examination during late summer and their mean concentrations were then 99 nmol/L. Determinants of serum 25(OH)D concentration during summer were baseline serum 25(OH)D concentration during winter, sun habits, body mass index and skin type.

From the ULSAM 833 men aged  $71 \pm 0.6$  years had mean plasma 25(OH)D concentrations of 69 nmol/L. They were examined in a cross-sectional study to determine the association between plasma 25(OH)D concentrations and the prevalence of hypertension. In a multivariable adjusted logistic regression model, men with plasma 25(OH)D concentrations  $<37.5$  nmol/L had a 3-fold higher prevalence (OR=3.3 (95% CI:1.0–11.0)) of hypertension compared to those with  $\geq 37.5$  nmol/L.

In a sub-group from SMC 550 women, aged 59-85 years had serum 25(OH)D concentration mean of 79 nmol/L. The women were examined using a simultaneous quantile regression model to estimate differences in percentiles of systolic, diastolic, mean arterial and pulse pressure in relation to serum 25(OH)D status. The multivariable adjusted statistically significantly difference in pulse pressure for the group of women with low serum levels of 25(OH)D ( $<50$  nmol/L) compared with high ( $\geq 100$  nmol/L) was 7.2 mmHg (95% CI: 2.5-11.9) within the 25<sup>th</sup> percentile of pulse pressure.

For the meta-analysis study-specific results were combined using a random-effects model. The summary odds ratios (95% CI) for hypertension comparing the highest versus the lowest circulating 25(OH)D concentrations were 0.73 (0.63-0.84), and 0.84 (0.78-0.90) for 40 nmol/L increment in the dose-response meta-analysis.

In conclusion, dietary vitamin D, travel to sunny location and the use of dietary supplements during winter seems to affect serum 25(OH)D concentrations and sun habits, body mass index and skin type during summer. Findings indicate that circulating 25(OH)D concentrations are associated with hypertension and pulse pressure.

## LIST OF PUBLICATIONS

- I. **Burgaz A**, Åkesson A, Öster A, Michaëlsson K, Wolk A  
Association of diet, supplement use and UVB exposure with vitamin D status in Swedish women during winter. *Am J Clin Nutr.* 2007;86:1399-404.
- II. **Burgaz A**, Åkesson A, Michaëlsson K, Wolk A  
25-hydroxyvitamin D accumulation during summer in elderly women at latitude 60° N. *J Intern Med.* 2009;266:476-83.
- III. **Burgaz A**, Byberg L, Rautiainen S, Orsini N, Håkansson N, Ärnlov J, Sundström J, Lind L, Melhus H, Michaëlsson K, Wolk A  
Confirmed hypertension and plasma 25(OH)D concentrations among elderly men *J Intern Med.* 2011;269:211-8.
- IV. **Burgaz A**, Orsini N, Håkansson N, Michaëlsson K, Wolk A  
Serum 25-hydroxyvitamin D concentrations in relation to blood and pulse pressure in elderly women *Submitted*
- V. **Burgaz A**, Orsini N, Larsson SC, Wolk A  
Blood 25-hydroxyvitamin D concentration and hypertension: a meta-analysis *J Hypertens.* 2011;29:636-45.

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## LIST OF ABBREVIATIONS

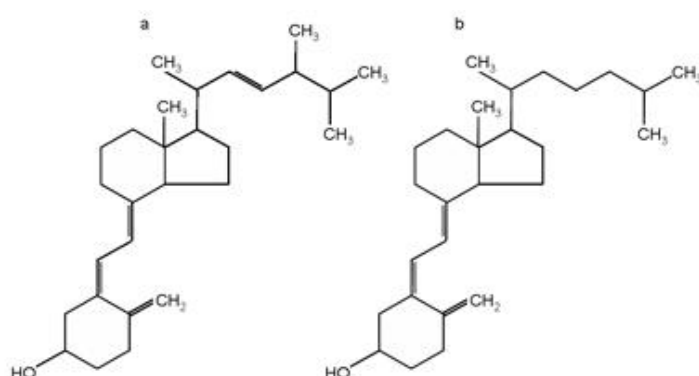
25(OH)D	25-hydroxyvitamin D, Calcidiol
1,25(OH)D	1,25-dihydroxyvitaminD, Calcitriol
ABPM	Ambulatory blood pressure monitoring
BP	Blood pressure
BMI	Body mass index (kg/m <sup>2</sup> )
CI	Confidence Interval
COSM	Cohort of Swedish Men
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DEQAS	Vitamin D External Quality Assessment Scheme
HPLC MS	High-pressure liquid chromatography mass spectrometry
IU	International unit
MAP	Mean arterial pressure
MED	Minimal erythematous dose
MLR	Multiple linear regression
µg	Microgram
mmHg	Millimeter mercury
ng	Nanogram
PP	Pulse pressure
PTH	Parathyroid hormone
RAS	Renin Angiotensin System
SBP	Systolic blood pressure
SMC	Swedish Mammography Cohort
SPF	Sun protection factor
ULSAM	Uppsala Longitudinal Study of Adult Men
UVA	Ultraviolet A
UVB	Ultraviolet B
VDBP	Vitamin D-binding protein
VDR	Vitamin D receptor
Vitamin D <sub>2</sub>	Ergocalciferol
Vitamin D <sub>3</sub>	Cholecalciferol

# 1 BACKGROUND

## 1.1 VITAMIN D

Vitamin D is the name of a group of fat-soluble compounds that are essential for maintaining the appropriate mineral balance in the body. Vitamin D, called the most important vitamin, was discovered in 1922 and at the time was primarily associated with bone health. The chemical structures of the D vitamins were determined in the 1930s by Professor Adolf Windaus's laboratory at the University of Göttingen in Germany and in 1971 Anthony W. Norman at the University of California, discovered the 1,25-dihydroxyvitamin D, the active form of vitamin D hormone.

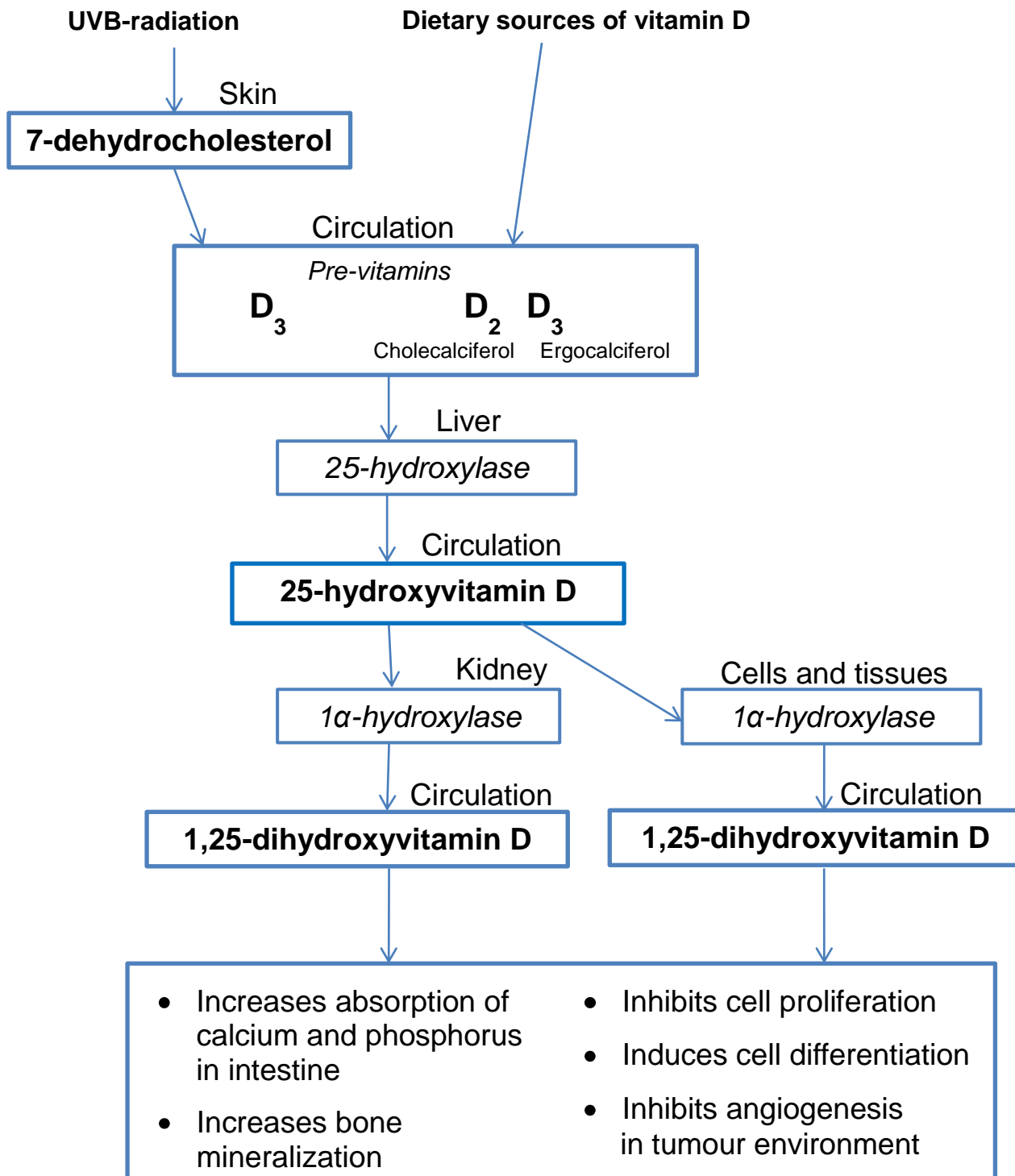
Vitamin D belongs to a group of several related sterols, with the two most important being D<sub>2</sub> (ergocalciferol) and D<sub>3</sub> (cholecalciferol) (Figure 1). The two forms differ chemically only in their side-chain structure, vitamin D<sub>2</sub> has a side chain that contains a double bond between carbon 22 and carbon 23 and a carbon 24 methyl group.



*Figure 1.*  
*a) D<sub>2</sub> (ergocalciferol)*  
*b) D<sub>3</sub> (cholecalciferol)*

Vitamin D<sub>3</sub> is produced by the skin as a result of ultraviolet (UV) irradiation of 7-dehydrocholesterol or by digestion of animal products. Vitamin D<sub>2</sub> is formed by UV radiation of the plant sterol ergosterol and humans can obtain vitamin D<sub>2</sub> only from plant products. The name calciferol, vitamin D, refers to both vitamin D<sub>2</sub> and D<sub>3</sub>.

Vitamin D is biologically inactive and requires metabolism in the liver on carbon 25 to form the main circulating form of vitamin D, 25-hydroxychole-calciferol or -calcidiol (25(OH)D). The compound 25(OH)D is used to measure vitamin D status. To be activated 25(OH)D must be converted to the form 1,25-dihydroxychole-calciferol or – calcitriol (1,25(OH)D) (Figure 2). Vitamin D-binding protein (VDBP) is the major carrier of vitamin D and its metabolites, 25(OH)D and 1,25(OH)D. The vitamin D receptor (VDR) is the mediator of the biological actions of 1,25(OH)D. Receptor polymorphisms, hormone status and several other factors influence possible effects of vitamin D (Holick 1995, Wolpowitz and Gilchrest 2006).



There is little known about pre vitamin D and 25(OH)D storage in the adult human body because of the lack of total carcass data for humans. The most complete pig carcass (90%) analysis indicated that the distribution of pre vitamin D and 25(OH)D was 2/3 and 1/3 respectively. In this analysis the storage distribution of pre vitamin D was 73% in fat tissue, 16% in muscles and 3% in serum. Regarding 25(OH)D 34% was stored in fat, 20% in muscles and 30% in serum (Heaney *et al.* 2009).

Figure 2. Vitamin D metabolism



## 1.2 DETERMINANTS OF VITAMIN D STATUS

### 1.2.1 UVB-induced vitamin D

#### 1.2.1.1 *Sunlight exposure*

Sunlight is composed of radiation of varying wavelengths, ranging from the infrared long wavelength light to the ultraviolet short wavelength light (UV). UV light is further divided into UVA (315 nm-400 nm) and the shorter wavelength UVB radiation. UVB causes sunburns, but it also initiates Vitamin-D production in the skin. The 7-dehydrocholesterol (pro-vitamin D), a cutaneous membrane lipid, absorbs UVB radiation between wavelengths of 280 and 315 nm and will thereby be converted into pre-vitamin D<sub>3</sub> (pre-cholecalciferol). Pre-vitamin D<sub>3</sub> will isomerize into vitamin D<sub>3</sub> (cholecalciferol) (Figure 2). Vitamin D<sub>3</sub> is transported to the liver by binding to VDBP. In elderly, the production capacity of vitamin D is lowered because of atrophy of the skin due to lower amount of membrane lipids (Barysch 2010).

Sun exposure does not result in overload quantities of the production of vitamin D. The reason is that pre-vitamin D<sub>3</sub> efficiently absorbs sunlight and is converted to many other photoproducts, including lumisterol, tachysterol, suprasterols, and toxisterols. Because of this regulation, the skin will never generate quantities of vitamin D<sub>3</sub> extreme enough to cause vitamin D intoxication (Holick 1994, Holick 2003, Holick, *et al.* 1995). Both UVA and UVB radiation increase the risk of skin cancer. UVA radiation intensity is the dominant source of radiation from the sun and is relatively constant, while UVB intensity varies with latitude, time of day, time of year and many other variables.

#### 1.2.1.2 *Latitude; especially Nordic*

The Northern European population may be at risk for vitamin D insufficiency, because of high latitude leading to limited UV light exposure (Figure 3). Latitude is often expected to be one of the most important factors influencing vitamin D status. At latitudes above 40° (the latitude of Sweden is 55°-69°), photo-conversion of 7-dehydrocholesterol to pre-vitamin D<sub>3</sub> does not occur in winter months, and as latitude rises, even summer synthesis is blunted (Barger-Lux and Heaney 2002). Vitamin D status, reflected by the level of 25(OH)D, is closely correlated with solar UV radiation (van der Wielen, *et al.* 1995, Webb and Holick 1988). At high latitudes circulating 25(OH)D concentrations vary between summer and winter months, following the seasonal variations in UV radiation with a delay (Figure 3).

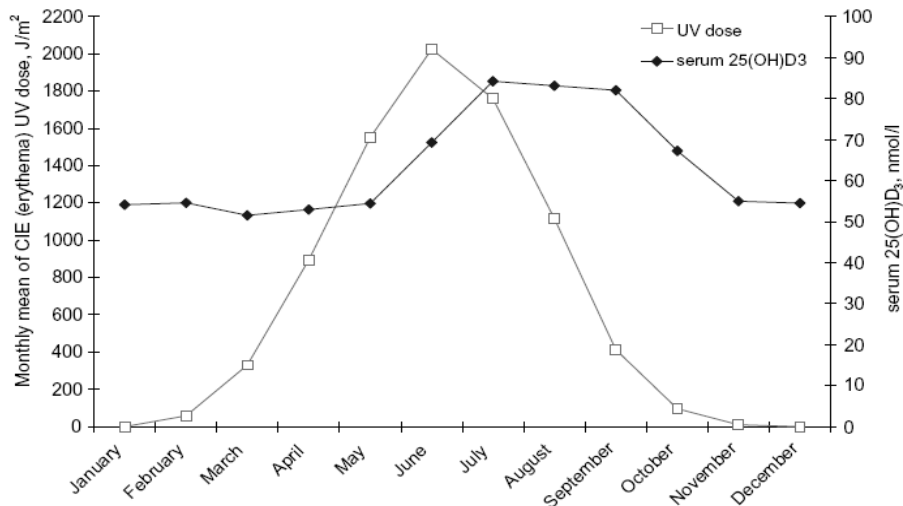


Figure 3. The monthly variation of vitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) in latitude 68°, together with monthly values of erythemogenic UV radiation, averaged over the years 1996-1999 (Robsahm, *et al.* 2004)

In general, the maximum serum concentration of vitamin D is observed to peak between August and October. Although the relative photosynthesis of vitamin D in the skin throughout the year in low latitude regions is higher than in high latitude regions due to more UVB radiation, living in sunny climates does not ensure 25(OH)D sufficiency (Kimlin 2004). Populations residing at higher latitudes in northern Europe are believed to be particularly vulnerable to reduced 25(OH)D levels (Calvo and Whiting 2005). In the Scandinavian latitudes, during winter, the cutaneous synthesis of pre-vitamin D is not detectable but sun exposure during the summer season does promote vitamin D synthesis in human skin (Chapuy, *et al.* 1997) (Figure 4). Sun protective habits of southern Europeans may have contributed to their lower vitamin D status than the Nordic Countries (Table 6, page 51). In addition, the nutritional supply of vitamin D is low in most countries and fortification of food is made in only a few countries, mainly in Scandinavia. The percentage of people taking vitamin D supplements is higher in Northern Europe compared to Southern Europe. This might explain why vitamin D insufficiency is more prevalent in Southern Europe (Table 6, page 51). Other factors such as time outside, clothing habits, and skin pigmentation also contribute to the differences in vitamin D status among countries (Park and Johnson 2005).

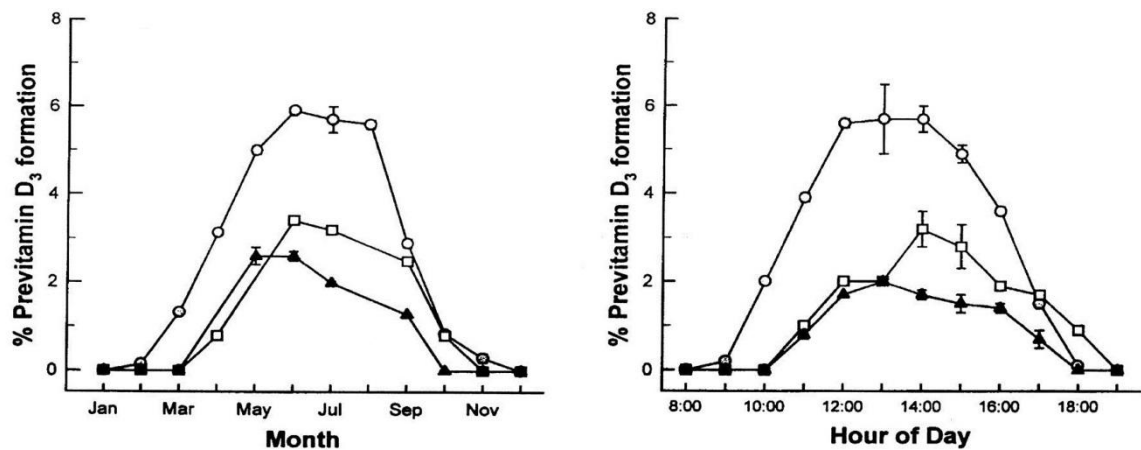


Figure 4. Influence of season, time of day, and latitude on the synthesis of pre-vitamin D<sub>3</sub> in the Northern hemisphere. Boston ○ Edmonton □, Bergen ► (Holick 2004)

#### 1.2.1.3 Tanning bed

Both the sun and tanning beds produce two different types of UV radiation, UVA and UVB. Our skin does not absorb these two types of radiation in the same way. UVA rays have longer wavelengths which penetrate down to the deepest layers of the skin, while UVB ray wavelengths are shorter and reach only the surface layers of the skin. Both UVA and UVB radiation contribute to health risks associated with excessive sun exposure, such as the risk of developing skin cancers. On the other hand, UVB radiation also activates the synthesis of the vitamin D precursor in the skin, 7-dehydrocholesterol, and is therefore responsible for the health benefits of sunshine. Exposure of arms and face to “real” daytime sunshine for on average 10-15 minutes/day at North latitudes, during summer season, provides the light skin type of a Caucasian with sufficient amount of UVB radiation to eliminate vitamin D deficiencies, without causing damage to the skin (Samanek 2006). While UVB radiation accounts for the health benefits of sunshine, the tanning salons are more interested in UVA radiation because too much exposure to UVB radiation affects the surface layers of the skin and quickly causes sunburns. New research proposes that UVA exposure might be as damaging to the skin as UVB (Skin Cancer Foundation). While it has been known for several years that UVA penetrates more deeply into the skin than UVB, it was assumed that less of the UVA was absorbed by DNA, causing fewer cancer mutations (Skin Cancer Foundation). UVA radiation contributes to the golden-brown tan required by persons using tanning beds. Therefore, most tanning salons regulate their tanning beds to produce approximately 95% UVA radiation. This regulation raises the bronzing effects of the tanning bed, but also increases the risk of burning. It also minimizes the quantity of vitamin D skin production in relation to the exposure to damaging UV radiation. A tanning bed can be regulated to

emit a greater amount of UVB radiation but the safety of exposure to either type of UV radiation depends on its balance. Because most people who enter a tanning bed expose a lot of their surface area of the skin, it could quickly result in an excessive absorption of UVB radiation even if the percentage of UVB radiation is quite low.

#### *1.2.1.4 Skin type*

Larger amounts of the pigment melanin in the epidermal layer result in darker skin and decrease the capacity of the skin to produce vitamin D from UVB radiation (Clemens, *et al.* 1982). Reports consistently indicate lower circulating 25(OH)D levels in black persons compared with Caucasians. However it is not clear whether lower levels of 25(OH)D in persons with dark skin cause health concerns.

The quantity of UVB radiation it takes for the body to produce a sufficient amount of vitamin D depends on how much skin is exposed and the skin type. The Fitzpatrick Classification Scale defines six skin types by complexion and tolerance of UV radiation (Fitzpatrick 1975). Low skin type number indicates that less time in the sun is required to make vitamin D. Dark skin, that never develops a sunburn, requires 10-50 times the exposure to sunlight to produce the same quantity of vitamin D in the skin, as does compared to pale skin (Clemens, *et al.* 1982). Exposure of the skin to one minimal erythemal dose (MED) (a slight pinkness of the skin), increases the circulating 25(OH)D concentrations to a level equivalent to ~250-500 µg (10 000-20 000 IU) oral vitamin D (Holick 2005). For pale skin, the exposure period for one MED in the summer sun during the middle of the day in the southern United States (latitude 30°) is about 4-10 minutes (Grant and Holick 2005). Depending on UVB radiation to meet vitamin D needs through endogenous synthesis is a question of current debate regarding the increased risk for skin cancer and the importance of optimal vitamin D status. An investigation is needed as to whether there is a threshold for meeting vitamin D needs through UVB exposure while the risk of skin cancer is at a minimum. The question is if it is possible to optimize UVB radiation exposure between skin cancer-risk and a healthy vitamin D status (Moon, *et al.* 2005).

#### *1.2.1.5 Sunscreen*

Sunscreens work by absorbing UVB radiation and also some UVA before it enters the skin. Studies have found that sunscreen limits the vitamin D production by blocking UV radiation. According to a few studies, this blocking can decrease the vitamin D production by as great as a tenfold (Matsuoka, *et al.* 1988). These studies reported that sunscreen with a sun protection factor (SPF) of eight reduces the capacity of the skin to

produce vitamin D by more than 95%. Correctly used sunscreen with a SPF of 15 reduces the capacity by more than 98% (Matsuoka, *et al.* 1987). More recent, randomized studies that followed people for months (Marks, *et al.* 1995) and years (Farrerons, *et al.* 1998) suggest that the effect is negligible. According to these studies, while sunscreen does block vitamin D production, it is not enough to cause a low vitamin D status. Presumably this occurs because most people do not apply enough sunscreen to get its full effect, which gives the sunlight the opportunity to get through to reach the 7-dehydrocholesterol in the skin. Therefore, sunscreen can reduce vitamin D production, but probably not enough to lead to vitamin D insufficiency.

#### *1.2.1.6 Age*

Elderly people in Europe, especially nursing home residents, often suffer from vitamin D insufficiency. Aging decreases the amount of 7-dehydrocholesterol produced in the skin by as much as 75% by the age of 70 years. Therefore, a 70 year old person has approximately 25% of the capacity to produce cholecalciferol compared with a healthy young adult (Holick, *et al.* 1989). However the skin has such a large capacity to make vitamin D that even elderly exposed to sunlight can achieve increased circulating concentrations of 25(OH)D.

#### *1.2.1.7 Obesity*

Obese individuals have been shown to have low circulating 25(OH)D concentrations (Bell, *et al.* 1985, Liel, *et al.* 1988). Since vitamin D is a fat soluble vitamin and is readily stored in adipose tissue, it could be sequestered in the larger body pool of fat of obese individuals. Researchers have observed that circulating 25(OH)D concentrations increased in both obese and lean subjects after exposure to an identical quantity of UVB radiation (Wortsman, *et al.* 2000). Obese subjects has a larger body surface area of exposure and would be expected to produce more pre-vitamin D which would result in higher circulating 25(OH)D concentrations, than would the lean subjects. However, the increase was less than half in the obese subjects than in the lean, one day after exposure. This indicates that the subcutaneous fat, which stores vitamin D, sequestered more of the cutaneous synthesized vitamin D in the obese than in the lean subjects since there was more fat available for this process. It has been suggested that obese individuals may avoid exposure to solar UV radiation, which is crucial for the cutaneous synthesis of vitamin D (Compston, *et al.* 1981).

### 1.2.2 Dietary vitamin D

The Swedish dietary vitamin D recommendations are 7,5 µg/day for adults and 10 µg/day for small children (<2 years) and the elderly (>60 years). Already some researchers in the field have suggested that the daily intake of vitamin D in countries with a low amount of UVB radiation should be above 15 µg/day and the recommended dietary allowances for those individuals are suggested to be raised to around 20-25 µg per day to secure a healthy level of 25(OH)D (Glerup, *et al.* 2000).

In USA the Institute of Medicine (IOM), which is part of the National Academy of Sciences, has been asked to update their recommendations for daily intake of vitamin D in the United States and Canada (IOM, 2010). A professional panel consisting of experts from both USA and Canada reviewed the latest research related to vitamin D. In the ground work for the recommendation they assume that blood levels of 50 nmol/L (20 ng/mL) is satisfactory. In November 2010, the committee released their new recommendations for dietary reference intakes of vitamin D. The new vitamin D recommendations in USA and Canada are twice as high as the Swedish, which were updated in 2004. With the new American guidelines there is already some controversy since many experts believe that the purposed recommended daily intake is still inadequate (Harvard School of Public Health, The Nutrition Source).

Among the elderly, vitamin D deficiency is unexpectedly more common in southern Europe than in Scandinavia. This could be explained by food fortification and vitamin D supplements that are common in Scandinavia (van der Wielen, *et al.* 1995). In Sweden it is obligatory to fortify non-fatty milk, non-fatty curdled milk, cooking oil and margarine. Currently most fortifying is done with vitamin D<sub>3</sub> from sheep's wool. According to the national survey "Riksmaten" (Dietary habits and nutrients' intake in Sweden), performed by Svenska Livsmedelsverket (SLV), the main dietary sources of vitamin D are margarines, fatty fish, shellfish and fortified milk-products. Sweden is among countries with the highest vitamin D intake in Europe (Freisling *et al.* 2010), but still we do not seem reach the recommended levels for vitamin D intake (Table 1). Sweden also have the highest circulating 25(OH)D concentrations in Europe in spite of the absence of UVB exposure during winter (Table 6, page 52).

#### 1.2.2.1 Food sources

In nature very few foods contain vitamin D. The flesh of fatty fish (e.g. salmon, herring, mackerel, sardines and tuna) and fish liver oils are among the best sources (USA Food and Nutrition Board 2010). Small amounts of vitamin D are also found in beef liver, cheese, and egg yolks. Vitamin D in these foods is primarily in the form of vitamin D<sub>3</sub> and its metabolite 25(OH)D<sub>3</sub> (Ovesen, *et al.* 2003). Some mushrooms

provide vitamin D<sub>2</sub> in variable amounts (Calvo, *et al.* 2004, Outila, *et al.* 1999). Mushrooms which have enhanced levels of vitamin D<sub>2</sub> from being exposed to UV light can be an alternative for non-eaters of foods of animal origin. Fishes and seafood provide 44.4% of the vitamin D in the Swedish Mammography Cohort (SMC) study and fortified products 36.1% (Figure 5).

*Table 1. Mean dietary vitamin D intake in representative samples of independent elderly subjects in some European countries (intake from supplements are not included, (Ovesen, et al. 2003).*

Country	Survey	Age	Intake (µg/d)			
			Men	n	Women	n
Sweden	Riksmaten 1 997–8 (SLV, Becker & Pearson, 2002)	>65	7.1	65	4.9	58
	SMC, (Burgaz et al, 2007)	61–86			6.0	116
Denmark	The Danish Dietary Survey, 1995 (Danish Food Agency, 1996)	65–74	3.3	103	4.1	122
		75–80	3.2	44	3.7	64
Norway	Norkost 1997 (Johansen & Solvoll, 1999)	60–69	5.6	131	4.0	137
		70–79	6.0	106	4.0	109
France	INCA 1999 (Volatier, 2000)	>65		2.5	245*	
Netherlands	The Third Dutch National Food Consumption Survey 1997–8 (Hulshof et al. 1998)	>65	4.8	185	3.6	236
Germany	Ernährungsbericht 2000 (Deutsche Gesellschaft für Ernährung, 2000)	65–74	3.7	361	3.1	503
		75–84	3.5	126	3.0	285
		>65	4.1	23	2.6	73
UK	National Diet and Nutrition Survey. People aged 65 years and over (Finch et al. 1998)	65–74	4.3	271	3.0	256
		75–84	3.8	265	3.0	217
		>85	3.2	96	2.3	170

*\*Intake for men and women combined*

#### *1.2.2.2 Vitamin D supplement*

Worldwide, supplements and fortified foods contain the two available forms of vitamin D, D<sub>2</sub> (ergocalciferol) and D<sub>3</sub> (cholecalciferol). Vitamin D<sub>2</sub> is manufactured by the UV radiation of ergosterol in yeast, and vitamin D<sub>3</sub> is manufactured by the radiation and the chemical conversion of 7-dehydrocholesterol from sheep lanolin (Holick 2007). Conventionally the two forms have been regarded as equivalent based on their ability as a treatment for rickets. Most steps involved in the metabolism and mechanisms of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> are identical. Both vitamin D<sub>2</sub> and vitamin D<sub>3</sub> effectively increase circulating 25(OH)D levels and it appears that at nutritional doses, vitamin D<sub>2</sub>

## Vitamin D sources

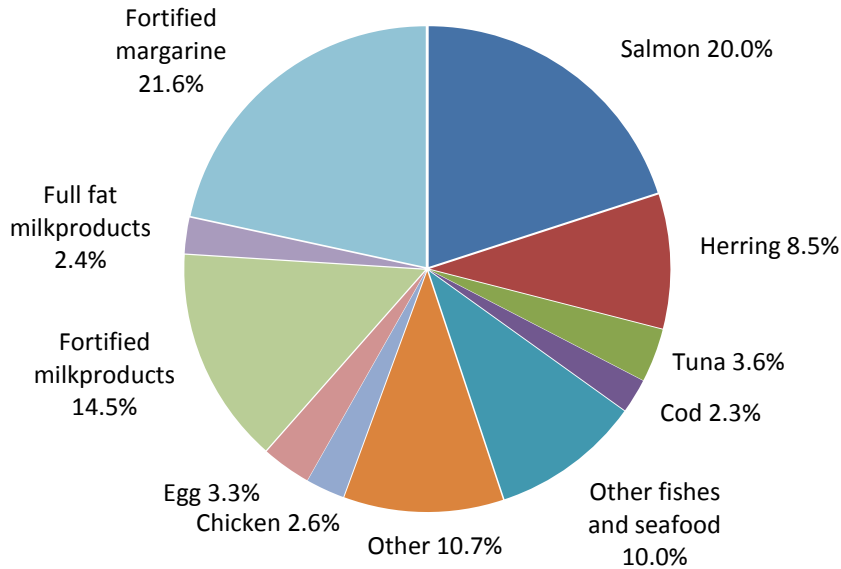


Figure 5. Dietary sources of vitamin D in 116 women aged 61-86 years from central Sweden.

and  $D_3$  are equivalent, but at high doses vitamin  $D_2$  is less potent. Vitamin  $D_3$  raises and maintains 25(OH)D levels to a greater degree than does vitamin  $D_2$  (Figure 6). Studies reporting 10  $\mu\text{g}$  supplement intake per day observed a mean increase in circulating 25(OH)D concentration of about 32 nmol/L (ranging from 18-45 nmol/L) (Vieth 1999). A systematic review of 16 trails reported that for intake of 2.5  $\mu\text{g}$  (100 IU) circulating 25(OH)D concentrations increased by 1-2 nmol/L (0.4-0.8 ng/mL) (IOM, 2010). Many factors do influence the increase of circulating 25(OH)D concentrations during a “vitamin D supplement study “ such as base 25(OH)D levels, age of subject, genetic factors, BMI, dietary vitamin D intake, sun exposure, dose and duration of vitamin D supplement.

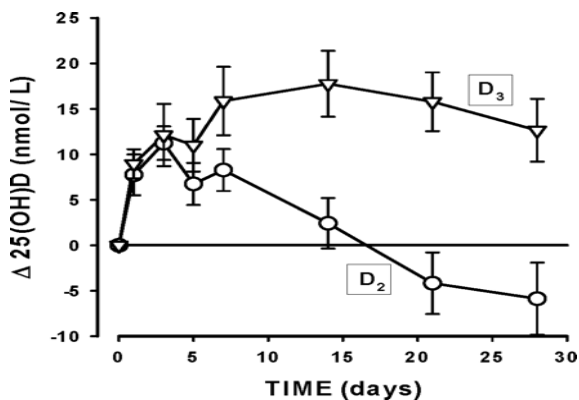


Figure 6. Time course of the rise in serum 25(OH)D after a single oral dose of either vitamin  $D_3$ , (cholecalciferol), or  $D_2$ , (ergocalciferol), to two equal groups (Armas et al. 2004)



### 1.2.3 Genetic factors

Worldwide variations observed in circulating 25(OH)D concentrations may be due to common environmental factors such as UVB exposure dependent on latitude, season, clothing related to religious or cultural issues, as well as diet, and fortified-food strategies. Individual behavioral aspects such as clothing, use of sunscreen, time spent outdoors, sun habits, use of vitamin D supplements, skin sensitivity and the body fatness may also affect concentrations (Calvo, *et al.* 2005, Holick 2007, Holick 2008). Individual and environmental factors as well as genetic predisposition could play a role in the possibility to increase circulating 25(OH)D levels. It is very important to further investigate the influence of genetic factors as compared with environmental factors on the vitamin D status. There are conflicting results regarding the genetic effect on summer and winter vitamin D status (Karohl, *et al.* 2010, Snellman, *et al.* 2009). Vitamin D concentrations could be influenced by genetic factors in several potential ways. First is individual skin sensitivity and the capacity to produce vitamin D<sub>3</sub>, which includes the presence of the substrate 7-dehydrocholesterol, the ability to convert 7-dehydrocholesterol to pre-vitamin D<sub>3</sub> and further to vitamin D<sub>3</sub>. Second is the catabolism of formed pre-vitamin D<sub>3</sub> into inactive vitamin D photoproducts such as lumisterol, tachysterol, suprasterols, and toxisterols. Third alternative genetic factor that may influence circulating 25(OH)D concentrations involves the vitamin D-binding protein (VDBP), which binds to vitamin D and its plasma metabolites and transports them to target tissues (Andreassen 2006, Speeckaert, *et al.* 2006). Another process possibly influenced by genes is the hydroxylation of 25(OH)D to 1,25(OH)D by the enzyme 1,α-hydroxylase, particularly since 1,α-hydroxylase has been found in almost all cells and tissues (Cheng, *et al.* 2004, Gupta, *et al.* 2004). During summer, 25(OH)D concentrations could be influenced by genetic factors such as the capacity to synthesize vitamin D in the skin (Snellman, *et al.* 2009). During winter, the lack of UVB radiation in the northern latitudes may make circulating 25(OH)D concentrations more dependent on the synthesis from dietary sources through all the different possible heritable pathways regulating vitamin D binding protein, the hydroxylation of vitamin D to 25(OH)D by the enzyme 25-hydroxylase and the release of vitamin D that might be stored in fat cells (Karohl, *et al.* 2010).

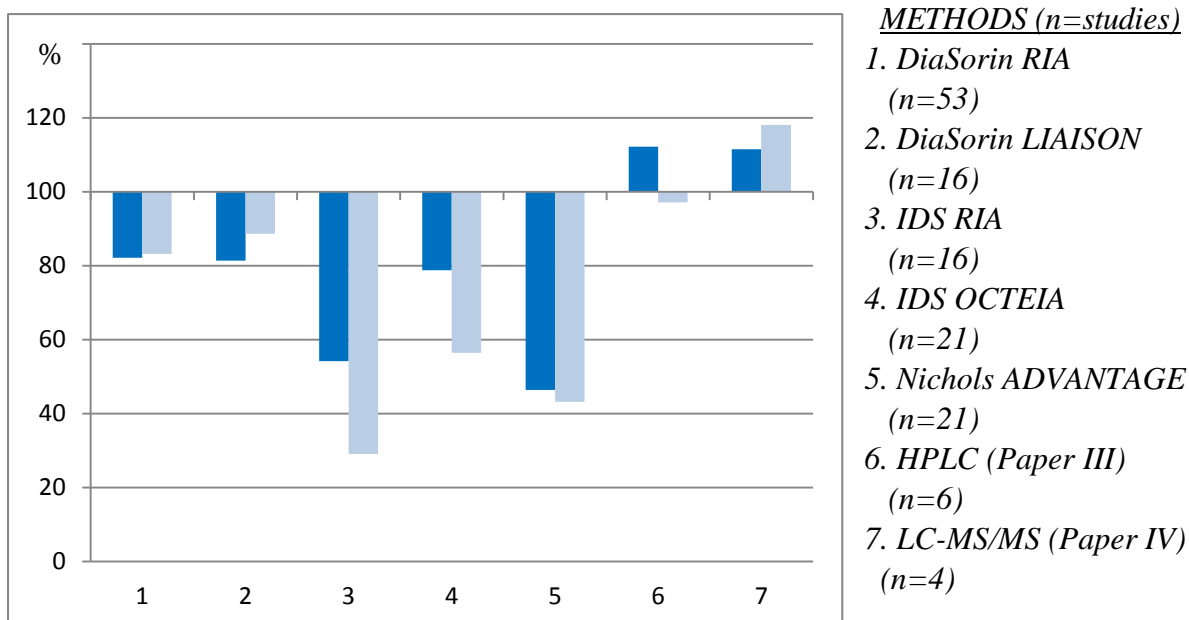
### 1.2.4 Assessment of circulating 25(OH)D concentrations

The interest regarding vitamin D health effects has been increasing and as a result the way of measuring vitamin D status has also increased. Still there is little consensus for which assay should be used measuring circulating 25(OH)D concentrations.

The International Vitamin D External Quality Assessment Scheme (DEQAS) sends out 5 samples four times a year to participating laboratories to establish all laboratory trimmed means (ALTM) and the method means with SDs. Laboratories with results meeting the ALTM criteria are awarded with certificates. There is also a possibility to occasionally participate in the evaluation to calibrate results. Results from 612 laboratories were collected in October 2009 and data from that distribution compared for the 7 most used methods (Figure 7) (Carter *et al.* 2006, Lai *et al.* 2010).

Circulating 25(OH)D in the Swedish Mammography Cohort (SMC) (n= 1041) and in the Uppsala Longitudinal Study of Adult Men (ULSAM) (n=1194) was shown to consist of ~99% of 25(OH)D<sub>3</sub> and only ~1% of 25(OH)D<sub>2</sub> according to LC-MS/MS and HPLC methods.

Figure 7. Comparison in percent between 7 different methods measuring mean 25(OH)D concentrations from one known 25(OH)D sample. The comparison refers to both circulating 25(OH)D<sub>3</sub> (blue) and 25(OH)D<sub>2</sub> (light blue)



All methods have advantages and disadvantages regarding economy, laboratory personnel knowledge, time consumption, amount of sample needed etc. For clinical practice it is important with a cut point to determine vitamin D deficiency; maybe they should consider using assay specific cut points in clinical practice (Lai *et al.* 2010).

### 1.3 THE ROLE OF VITAMIN D IN HEALTH

In epidemiological studies vitamin D has been suggested to prevent several diseases. While it may not be a cure, a deficiency in vitamin D may be a risk factor for disease and therefore the increase in the number of individuals being diagnosed with vitamin D deficiency can be a public health problem.

#### 1.3.1 Bone health

The steroid hormone 1,25(OH)<sub>2</sub>D gets transported by the VDBP to its target organs which control calcium and phosphorus metabolism. The interaction between 1,25(OH)<sub>2</sub>D and its nuclear vitamin D receptor (VDR) in the small intestine increases the expression of calcium channels and calcium binding protein which results in increased absorption of calcium from the diet (Christakos, *et al.* 2003, Holick 2003). Vitamin D sufficiency will activate the calcium transport system and permits dietary calcium to be absorbed into the bloodstream. A low dietary intake of calcium will increase the secretion of parathyroid hormone (PTH), which improves renal production of 1,25(OH)<sub>2</sub>D, thereby increasing the efficiency of calcium absorption. 1,25(OH)<sub>2</sub>D will also increase the absorption of dietary phosphorus. Approximately 55–70% of dietary phosphorus is passively absorbed. Vitamin D increases phosphorus absorption by an additional 20% so that approximately 80% of dietary phosphorus is absorbed (Holick 2007). The main function of vitamin D is to uphold serum calcium within a satisfactory range for the maintenance of neuromuscular function, signal transduction and a wide variety of metabolic processes (Holick 2003, Steingrimsdottir, *et al.* 2005). In infants and children, heavy vitamin D deficiency results in failure of bone mineralization. Rapidly growing bones are at the greatest risk to be affected by rickets. The growth plates of bones continue to broaden, but in the absence of satisfactory mineralization, arms and legs become deformed. In infants, the result of rickets might be delayed closure of the fontanelles in the skull, and the rib cage may become deformed because of the pulling action of the diaphragm. Although vitamin D fortification of foods has led to decrease in vitamin D deficiency in most developed countries, nutritional rickets is still being described in places all over the world (Wagner and Greer 2008, Wharton and Bishop 2003). Adult bones that are no longer growing are still in a constant state of turnover, or “remodeling”. The collagenous bone matrix is maintained but bone mineral is gradually lost, causing bone pain and osteomalacia (soft bones) in adults with severe vitamin D deficiency. Although osteoporosis is a multifactorial disease, vitamin D insufficiency can be a very important contributing factor. In an international survey of 18 different countries, ranging from latitude 64 in north to latitude 38 in south, including more than 2 600 postmenopausal women with osteoporosis, it was discovered

that 64% of subjects had circulating 25(OH)D concentrations lower than 75 nmol/L (30 ng/mL) (Lips, *et al.* 2006). However, the Randomized Evaluation of Calcium Or vitamin D (RECORD) trial reported that oral supplemental vitamin D<sub>3</sub> alone, or in combination with calcium, did not prevent the incidence of osteoporotic fractures in elderly adults who had a previous experienced low-trauma or osteoporotic fracture (Grant, *et al.* 2005). Lack of an effect could be due to a low compliance in this study or the fact that vitamin D supplementation (5-15µg, 200-600 IU vitamin D/day) did not increase serum 25(OH)D concentrations to a level that would be protective against fractures (Bischoff-Ferrari, *et al.* 2006). Although, vitamin D supplements without calcium seem to be less effective in fracture prevention (Abrahamsen *et al.* 2010). A study that reported the largest amount of vitamin D supplement used in a randomized trial (12 500 µg, 500 000 IU) showed higher risk for fractures among those who got supplement, indicating future safety with such high doses (Sanders *et al.* 2010). Clinical trials have generally found that vitamin D<sub>2</sub> is not effective at preventing fractures (Houghton and Vieth 2006).

### **1.3.2 Cancer**

Two characteristics of cancer cells are the lack of cell differentiation and rapid uncontrolled growth or proliferation. Most malignant tumors, including breast, lung, skin, colon, and bone tumors, have been discovered to contain VDR. The biologically active form of vitamin D, 1,25(OH)D and its analogs, have been found to induce cell differentiation and suppress proliferation of a number of cancerous and noncancerous cell types preserved in cell culture (Blutt and Weigel 1999). The worldwide distribution of for example colon cancer mortality shows a similar pattern as the historical geographic distribution of rickets (Garland, *et al.* 1999, John, *et al.* 1999), providing some evidence that low sunlight exposure and vitamin D status might be related to an increased risk of colon cancer. More recent studies have reported that greater vitamin D intakes and circulating 25(OH)D concentrations are associated with decreased colorectal cancer risk. A five-year prospective study with more than 120 000 participants, reported that men with the highest vitamin D intake had a 29% decreased risk of colorectal cancer compared to men with the lowest vitamin D intakes (McCullough, *et al.* 2003). Another study with pooled, dose-response analysis of two case-control studies showed that women with breast cancer had significantly lower circulating 25(OH)D concentrations compared to the controls (Bertone-Johnson, *et al.* 2005, Lowe, *et al.* 2005). Another study reported that women with circulating 25(OH)D concentration of ~130 nmol/L (52 ng/ml) had a 50% lower risk of developing breast cancer compared to women with 25(OH) D levels lower than 32.5 nmol/L (13 ng/mL) (Garland, *et al.* 2007). In a prospective study from Finland, Norway and Sweden, a U-shaped relationship between serum 25(OH) D levels and prostate cancer

risk was observed; circulating 25(OH)D concentrations of 19 nmol/L (7.6 ng/ml) or lower and 80 nmol/L (32 ng/ml) or higher were associated with higher prostate cancer risk (Tuohimaa, *et al.* 2004). Epidemiological studies have demonstrated an association between risk factors for prostate cancer and environmental conditions that can result in low vitamin D levels (Blutt and Weigel 1999). There is a higher incidence of prostate cancer in African American men than in white American men, and the higher amount of melanin content in dark skin is known to reduce the efficiency of vitamin D synthesis (Staud 2005). In contrast to previous studies mentioned here there was no association observed between circulating 25(OH)D and cancer mortality in a recent study among elderly American men (Cawthon *et al.* 2010).

### **1.3.3 Autoimmune diseases**

Data from human, animal, and in vitro experiments are suggesting that vitamin D might play an important part in the autoimmunity (Cantorna and Mahon 2004). There is accumulating evidence of vitamin D status as a potential environmental factor affecting autoimmune disease prevalence. An unhealthy vitamin D status has been implicated in the etiology of autoimmune diseases such as multiple sclerosis (MS) (Ascherio, *et al.* 2010), rheumatoid arthritis (RA) (Cutolo *et al.* 2007), diabetes mellitus (DM) (Hyppönen 2010), and inflammatory bowel disease (IBD) (Cantorna 2006). It is clear that both genetic and environmental factors affect the prevalence of above mentioned diseases. Vitamin D has been involved as a factor in many different autoimmune diseases which suggest that vitamin D might be an environmental factor that normally participates in the control of the “self-tolerance” where the body does not mount an immune response to self-antigens. In addition, there may be a greater vitamin D requirement for patients at risk for developing or already having an autoimmune disease. The ideal amount of vitamin D to support the immune system may be different from the amount of vitamin D that is required for prevention of other diseases or to maintain calcium homeostasis. The severity of MS has been shown to vary seasonally, with worsening occurring mostly during spring (Bamford, *et al.* 1983, Goodkin and Hertsgaard 1989). Circulating levels of 25(OH)D concentrations also fluctuate seasonally, with decreased levels in late winter months and higher levels during late summer months. It seems reasonable that a lag time may exist between the dip in 25(OH)D levels and the worsening MS episode. The hypothesis that high intake of vitamin D is associated with the reduced risk of developing DM, RA and MS have been supported by some large prospective studies regardless of sunlight exposure (Cantorna and Mahon 2004). In addition to the data that indicate vitamin D status as an environmental factor that affects autoimmune disease prevalence, patients with autoimmune diseases also have been shown to express genetic polymorphisms for vitamin D regulatory genes. Polymorphisms in

the VDR have been associated with increased susceptibility to MS (Vitale, *et al.* 2002), RA (Garcia-Lozano, *et al.* 2001) DM (Motohashi, *et al.* 2003) and IBD (Martin, *et al.* 2002).

#### **1.3.4 Cardiovascular diseases**

The etiology of cardiovascular diseases (CVD) is still not totally understood. Mounting evidence suggests that vitamin D deficiency is associated with increased risk of cardiovascular diseases, but the underlying mechanisms remain to be explored in detail (Lee, *et al.* 2008). Data from epidemiologic studies indicate that geographic latitude, altitude, season, and the place of living are all associated with CVD mortality (Zitterman *et al.* 2005). There have been no adequate explanations offered for these coincidences. However, these environmental factors all share the property of influencing UVB exposure and therefore also human vitamin D status. The vitamin D hypothesis regarding the etiology of CVD is in line with the higher prevalence of CVD in obese and elderly individuals and the low prevalence of CVD in physically active individuals, since vitamin D status is inversely associated to body weight (Arunabh, *et al.* 2003, Wortsman, *et al.* 2000) and age (McKenna 1992, Passeri, *et al.* 2003) and is positively associated to the level of physical activity (Zittermann, *et al.* 2000). Also ethnicity influence vitamin D status and CVD risk (Grant *et al.* 2010). A systematic review show a statistically significantly inverse association between circulating 25(OH)D concentrations and cardiovascular disease among nine prospective studies regarding both CVD incidence and mortality, however there was a heterogeneity among the studies (Grandi *et al.* 2010). The heterogeneity could be due to different ethnicities among participants in the included studies. Ethnicity influence vitamin D status, one reason is differences in skin color and could be a reason for heterogeneity (Burgaz *et al.* 2010).

There are a number of mechanisms that might clarify the association between vitamin D status and CVD, among them an association of vitamin D with inflammatory markers. Activity of 1 $\alpha$ -hydroxylase is regulated by several inflammatory and hormonal mechanisms which suppresses 1 $\alpha$ -hydroxylase activity (Holick 2007). 1 $\alpha$ -hydroxylation of 25(OH)D occur in most cells and tissues in the body, nevertheless circulating concentrations of 1,25(OH)D are mainly predicted by renal 1 $\alpha$ -hydroxylase activity. The VDR is nearly ubiquitously expressed, and almost all cells respond to 1,25(OH)D exposure; about 5% of the human genome is regulated, directly and/or indirectly, by the vitamin D endocrine system (Zella *et al.* 2008). This suggests a widespread function and possible causal relationship between vitamin D deficiency and cardiovascular risk (Bouillon, *et al.* 2008, Lee, *et al.* 2008).

## **1.4 BLOOD PRESSURE AND HYPERTENSION**

### **1.4.1 Blood pressure**

Arterial blood pressure (BP) is dependent on two factors, the strength with which the heart pumps blood and the peripheral resistance in the blood vessels, mainly arterioles. Therefore, cardiac output and peripheral resistance are the two determinants of arterial pressure, and so BP is normally dependent on the balance between cardiac output and peripheral resistance (Cardiovascular Physiology Concepts). Cardiac output is determined by stroke volume and heart rate; stroke volume is related to myocardial contractility and to the size of the vascular compartment. Peripheral resistance is determined by functional and anatomic changes in small arteries and arterioles. Arterial pressure is read as for example, 120/80 and measured in mmHg. The peak pressure in the arteries is the systolic pressure and the minimum pressure in the arteries is the diastolic pressure. Individual BP may be affected by factors such as age, heart disease, emotions, activities, body position etc. (Cardiovascular Physiology Concepts).

### **1.4.2 Assessment of blood pressure**

#### *1.4.2.1 Office blood pressure*

The normal level for office BP is 120/80 mmHg or below. Office BP of 140/90 or above is considered as hypertension. BP is commonly measured in the clinical setting by a nurse or physician, and repeated clinic or office BPs are used as a basis for treatment decisions and evaluation of BP control. However, office BP monitoring has several limitations (Staessen *et al.* 1999). Problems that may affect the precision of the measurement include observer bias such as terminal digit preference and measurement error. There is also a possibility that BP measured at the office is not representative of the “true” BP that occurs normally and that some subjects experience an emotional response to the clinic environment, termed “white-coat hypertension”. “White coat hypertension” is a very common condition, occurring in up to 20% of patients. Additionally, close to one in ten people have a less well understood condition known as “masked” hypertension, in which BP readings are normal in the medical setting but sporadically high in real life (O'Brien 2008). Office-based BP readings are therefore limited regarding the amount of information they provide, as they represent a single snapshot in time. The limitations also include low reproducibility because the BP level in an individual is not constant, but continuously fluctuates over time, depending on both internal and external factors.

#### *1.4.2.2 Ambulatory blood pressure monitoring (ABPM)*

Accurate BP measurement is essential for diagnosis, treatment, and monitoring of hypertension. It has been clear that conventional office-based BP readings are not as truthful as ambulatory BP monitoring (ABPM) (Floras 2007). ABPM is a noninvasive method of BP measuring over 24 hours, whilst the patient is in their own environment, representing the true reflection of their BP. ABPM monitoring eliminates the “white-coat” effect and the “masked hypertension” effect and therefore provides more comprehensive information about the “true” BP level during all 24 hours of the day. ABPM is still not used very often in clinical practice although reference values of normal 24-hour BP have recently been proposed (O’Brien 2003). ABPM should be used especially in certain subgroups according to the current guidelines including borderline readings in clinic, poorly controlled hypertension (suspected drug resistance), patients who have developed target organ damage despite control of BP, pregnant women who develop hypertension, high risk patients, suspected “white coat” syndrome or “masked hypertension” and elderly patients with systolic hypertension (World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension). Elderly people may always benefit from ABPM because they often display variability in their BP which may obscure the office BP measurement. Misclassification of hypertension leading to unnecessary medication is harmful especially in older patients. Downsides to the ABPM are background noise (which may lead to interference) and some patients find the inflation of the cuff causes unbearable discomfort. The results provided from ABPM might vary according to the machines used. Additionally, it is not widely available and requires specially trained clinicians or nurses.

#### *1.4.2.3 Pulse pressure (PP)*

There is increasing interest regarding the association between BP variables and CVD risk in the general population, with a particular focus on pulse pressure (PP). PP is the difference between systolic blood pressure (SBP) and diastolic blood pressure (DBP). Accumulating evidence indicates that PP may be an important predictor of cardiovascular events (Lee, *et al.* 1999) especially in elderly persons (Chae, *et al.* 1999). Several large prospective trials as well as the re-analysis of previously collected data have convincingly demonstrated that the higher the PP level the greater the incidence of mortality in both normotensive and hypertensive subjects (Benetos, *et al.* 1998). Pulse pressure (PP) rises markedly after 50 years of age due to arterial stiffening with age (Franklin, *et al.* 1997). Some have proposed that PP is the best predictor of cardiovascular event (Nawrot, *et al.* 2004), whereas others have not (Kannel, *et al.* 1976). With advancing age, there is a progressive increase in SBP while the epidemiological data suggest that BP components may influence



cardiovascular risk differently at different ages, shifting from DBP being of importance before 50 years of age, to SBP and PP after age 60. DBP plateaus or even decreases after the age of 60 (Burt, *et al.* 1995) resulting in a wider PP. In the elderly the elastic properties of the large arteries decrease, as the amount of elastin is reduced while the proportion of collagen in the arterial wall is increased. The consequence of these degenerative changes is thickening, hardening and dilatation of the aorta, carotids and other large elastic arteries, whereas the smaller peripheral arteries contain less elastin, and therefore are more stable. The structural changes of the large arteries lead to a reduced elasticity, and an increased velocity by which the pulse wave is transmitted from the left ventricle through the arterial tree, causing an early backward return of the pulse wave from the periphery. This pulse-wave reflection causes an increase of the pressure during late systole, and a subsequent increase in SBP and decrease in DBP (O'Rourke 1999). PP, a simple relation with vessel stiffness, is associated with left ventricular hypertrophy. Increased PP has also been implicated in the development and progression of large-vessel atherosclerosis and small-vessel disease (Christensen 1991).

#### *1.4.2.4 Mean arterial pressure (MAP)*

The arterial system requires constant and adequate pressure to supply all of the organs with oxygenated blood. Very low BP will result in insufficient perfusion of organs and very high BP might cause CVD. It is important that BP is maintained within the range of levels that is required by tissues. For supplying the organs with enough oxygen and nutrients MAP should be 70-110 mmHg. If the value falls lower it means there is not sufficient amount of blood pumping into the organs. A high MAP indicates an increased cardiac output. MAP is affected by the blood volume pumped by the heart (cardiac output), heart rate, BP and the resistance to blood flow in the vessels. Abnormal levels of these factors can have an impact on MAP and effects the perfusion of organs like the brain and kidneys.

#### *1.4.2.5 Hypertension*

Hypertension is defined as BP above 140 mmHg systolic and/or 90 mmHg diastolic. BP is usually measured only once in epidemiological studies which might overestimates the prevalence of hypertension. Although, BP measured on one occurrence is preferable than self-reported hypertension which underestimates the prevalence of hypertension. Essential hypertension is the form of hypertension that by definition has no identifiable cause. It is the most common type of hypertension, affecting 95% of hypertensive patients (Carretero and Oparil 2000). Essential hypertension tends to be hereditary and is likely to be the consequence of an interaction

between environmental (for example vitamin D status) and genetic factors. Recent research shows that hypertension seems to be highly heritable and polygenic with a few candidate genes postulated in the etiology of this condition (Sagnella and Swift 2006).

Worldwide, the total estimated number of adults with high BP is approximately one billion, a number that is expected to grow dramatically. Hypertension is prevalent in developing as well as in developed countries (Kearney, *et al.* 2005) and thus is major public health challenge (He and Whelton 1997, Whelton 2004). Also, it is the most important transformable risk factor for cardiovascular, cerebrovascular and renal disease. Hypertension has been identified by the Risk Assessment Collaborating Group as the leading global risk factor for mortality and as the third leading risk factor for disease burden (Ezzati, *et al.* 2002). Whereas hypertension is well documented as a major cause of morbidity and mortality in the economically developed world, the importance of hypertension in non-developed countries is less well established.

Hypertension is a complex disease and the etiology of hypertension varies widely amongst individuals within a population (Dickson and Sigmund 2006). Hypertension is a major risk factor for coronary heart disease and stroke (Meredith and Ostergren 2006). There is a strong, continuous and graded association between BP and cardiovascular disease, but there is no clear threshold value that separates hypertensive patients who in the future will experience cardiovascular problems from those who will not. The risk of cardiovascular disease is influenced by BP and whether there is hypertensive damage to target organs. Many other factors are certainly involved in predicting cardiovascular risk, such as age, family history, sex, high cholesterol, smoking, diabetes, obesity, lifestyle habits, and left ventricular hypertrophy (Padwal, *et al.* 2001). The major risk determinant in younger populations is the DBP component, and in the elderly the SBP component (Franklin 2008). There are also some gender differences in cardiovascular risk and mortality, but they are not yet thoroughly investigated. Overall, cardiovascular morbidity is nearly three times higher in hypertensive men (Li, *et al.* 2006), and occurs earlier in men than in women. Age seems to be of greater importance in women and the risk of hypertension increases seriously after menopause (Mancia, *et al.* 2007).

## 1.5 VITAMIN D AND BLOOD PRESSURE

BP follows a seasonal variation through the year (Rostand 1997). Vitamin D also varies during the year, mainly following sun exposure. Vitamin D is possibly one of the factors affecting in the seasonal variation of BP. Vitamin D modulates the serum level of parathyroid hormone, which in association with calcium metabolism could influence the BP level. The discovery that 1,25(OH)D suppresses renin gene expression may explain, at least in part, the observed inverse relationship between vitamin D and BP (Rostand 1997).

### 1.5.1 Mechanisms

#### 1.5.1.1 *The renin-angiotensin system (RAS)*

Vitamin D regulates the gene expression of several genes that play a important role in the progression of heart failure, such as cytokines and hormones (Meems *et al.* 2011) Vitamin D is a negative regulator of the hormone renin, the essential hormone of the RAS (Li, *et al.* 2002, Qiao, *et al.* 2005). Increased activation of the RAS, which is a main regulator of electrolyte and volume homeostasis, contributes to the development of arterial hypertension (Connell, *et al.* 2008). Mechanistic insights have been gained by studying mice deficient for the vitamin D receptor, which develop hypertension and adverse cardiac remodeling mediated via the renin-angiotensin system (Li, *et al.* 2002, Simpson, *et al.* 2007, Xiang, *et al.* 2005, Zhou, *et al.* 2008).

#### 1.5.1.2 *Effects on cells of the vessel wall*

Vitamin D and its equivalents cause several effects on the cells of the vessel wall, which include vascular smooth muscle cells, endothelial cells and macrophages, all of them expressing the VDR as well as 1 $\alpha$ -hydroxylase (Bouillon, *et al.* 2008, Holick 2007, Peterlik and Cross 2005). Vitamin D's effect on vascular smooth muscle cells is complex and is also modulated by other hormones, such as parathyroid hormone and estrogenic compounds, which up-regulate 1 $\alpha$ -hydroxylase in these cells (Somjen, *et al.* 2006, Somjen, *et al.* 2005). 1,25(OH)D is thought to protect against vascular problems by decreasing endothelial adherence molecules, by increasing the activity of endothelial nitric oxide synthase, and through its anti-inflammatory properties (Talmor, *et al.* 2008, Talmor, *et al.* 2008).

#### 1.5.1.3 Vitamin D and blood pressure in randomized controlled trials (RCT)

Findings from studies investigating the effect of vitamin D supplementation on BP are inconsistent. This may be due to differences in the doses and duration of vitamin D supplementation used in the studies. Thirteen randomized trials have reported the effects of vitamin D<sub>2</sub> or vitamin D<sub>3</sub> supplementation on BP (Vaidya, *et al.* 2010). However, only two of the 13 trials were specifically designed to investigate the effect of vitamin D supplementation on BP. In those two studies antihypertensive medication was not permitted and the primary end-point was BP. Two meta-analyses have investigated the pooled results for vitamin D supplementation and BP. The first (Witham, *et al.* 2009) analyzed trials that had investigated vitamin D<sub>2</sub>, vitamin D<sub>3</sub>, alphacalcidol (a 1,25(OH)D analog), and/or UVB radiation as exposures. When the authors limited the investigation to four trials in which hypertensive participants had been given un-activated forms of vitamin D (D<sub>3</sub>; 20-72.5 µg, 800-2 900 IU, D<sub>2</sub>; 45 µg, 1 800 IU, UVB exposure; 6 weeks during February to March, irradiation commenced with an exposure time of 6 minutes at 0.7 of the MED) as a supplement, the pooled estimate regarding SBP was -6.2 mmHg (statistically significant) when pooling trials with only normotensive participants they observed no effect. The other meta-analysis (Pittas, *et al.* 2010) overall observed no significant effect of vitamin D supplementation on BP. Only when trials that used >1000 IU (25 µg) /day dose of vitamin D were included, there was a statistically significant small decrease of 1.5 mmHg in DBP. There are large differences in the dose of vitamin D supplementation used in randomized trials. An optimal dose required for increase of circulating 25(OH)D concentrations to an acceptable level has yet to be established. In addition, any differences in the effect of vitamin D from sun exposure and that from dietary sources remain to be characterized. Very high-single doses of vitamin D need to be evaluated as these may temporarily suppress levels of 1,25(OH)D with negative consequences (Vaidya and Forman 2010). Vitamin D pharmacology is very complex and therefore the best approach for optimizing vitamin D supplementation is hard to establish. Differences in the doses and duration of vitamin D supplementation used in the studies make the interpretation of findings complex. In summary, the mixed findings from RCT may in part be explained by factors such as insufficient power, design issues and differences in dose and duration of vitamin D supplementation.

## 2 AIMS

The overall objective of this thesis was to evaluate the association between circulating 25(OH)D concentrations and BP in both elderly men and women. Furthermore, to examine the determinants of circulating 25(OH)D concentrations in a representative population of middle-age, elderly and old Swedish women both during winter and summer season.

### **The specific aims were:**

- To investigate the relative importance of dietary intake of vitamin D, vitamin D from supplements and from UVB exposure to the serum concentration of 25(OH)D during winter in a general middle-age, elderly and old female population from central Sweden.
- To explore how dietary intake of vitamin D, vitamin D from supplements and from UVB exposure affect serum 25(OH)D concentrations during summer in the same Swedish female population.
- To investigate the relation between plasma 25(OH)D concentration and the prevalence of hypertension in a general elderly male population from central Sweden.
- To examine whether levels of SBP and DBP, PP and MAP are predicted by serum 25(OH)D concentrations in a middle-age, elderly and old Swedish female population.
- To quantitatively summarize, using meta-analysis, the accumulated evidence on the association between circulating 25(OH)D concentrations and hypertension.

## **3 MATERIALS AND METODHS**

### **3.1 STUDY POPULATIONS**

The papers (except paper V) in this thesis are based on two epidemiological studies:

- Swedish Mammography Cohort (SMC), paper I, II and IV
- Uppsala Longitudinal Study of Adult Men (ULSAM), paper III

### **3.2 SWEDISH MAMMOGRAPHY COHORT (SMC)**

From 1987 to 1990, all 90 303 women who lived in Uppsala County of central Sweden and were born between 1914-1948 and all women who lived in Västmanland County and were born between 1917-1948 received an invitation by mail to participate in a mammography screening program. A total of 66 651 (74%) women returned a completed questionnaire on diet, weight, height and education (Figure 8).

In 1997, a follow-up questionnaire was sent to all 56 030 cohort members who were still living in the study area; the follow-up questionnaire was extended to include information on physical activity, medical history, age at menarche, history of oral contraceptive use, age at menopause, postmenopausal hormone use, and lifestyle factors, cigarette smoking history and use of dietary supplements. A completed questionnaire was received from 38 984 women (70% response rate).

During 2008-09, the exposure information was updated and extended by sending out two new questionnaires – one questionnaire on health including several signs of symptoms of disease, sleeping, social relations etc. (2008) and one on diet, dietary supplement use, physical activity and other lifestyle factors (2009).

#### **3.2.1 SMC sub-cohort**

During 2003-2009 a subgroup of 8 311 women from the SMC cohort was invited for physical examination at Samariterhemmet in Uppsala city (5 022 participated, 60% participation rate). The examination was conducted out after an overnight fast and included bone mineral density measurement (DXA), sampling of blood, urine and fat tissue, height and weight measurements and a detailed questionnaire on diet and lifestyle factors. Body mass index (BMI) was calculated as weight divided by height squared. Starting in 2006 the participants also completed a questionnaire about sun exposure (Figure 9) and from 2007 their blood pressure (BP) was measured.

From 12 January to 10 March, 2006, 122 women from the cohort were contacted and 118 agreed to participate in the study about vitamin D (response rate 97%, paper I). Women participating in the study during winter were invited again during and after the summer (3 August to 29 September) of 2006 (response rate 86%). When comparing values from the 1997 questionnaire, participants in study I and II (year 2006) had a mean BMI of 24.7 kg/m<sup>2</sup> and the 550 participants in study IV (year 2008) had a mean of 24.3 kg/m<sup>2</sup>. All women in the SMC had a mean BMI of 25.0 kg/m<sup>2</sup> in the 1997 questionnaire.

Written informed consent was obtained from all participants. The Ethics Committees of the Karolinska Institutet approved the study.

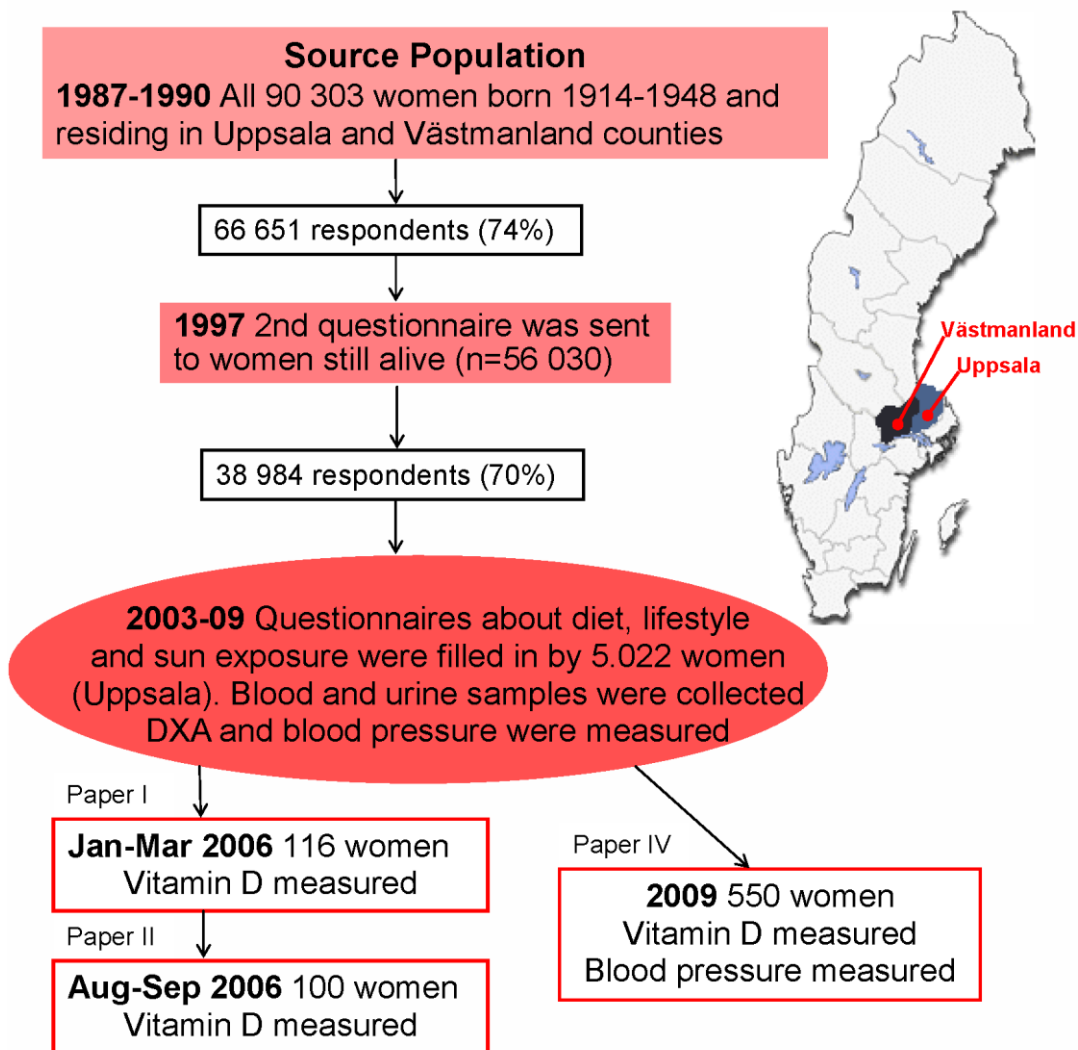


Figure 8. Study population for paper I, II and IV

#### 3.2.1.1 25(OH)D measurement

*Paper I and II.* Venous blood samples were drawn after an overnight fast and stored at  $-80^{\circ}$ . To evaluate vitamin D status, circulating 25(OH)D concentrations were measured in serum using an enzyme immunoassay (IDS OCTEIA Ltd., Boldon, UK). The assay quantifies 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> and the cross-reactivity to 25(OH)D<sub>3</sub> is 100% and to 25(OH)D<sub>2</sub> 75%. The immunoassay is based on highly specific sheep antibodies binding to vitamin D. To establish analytic quality, all standards, controls and samples were analyzed in duplicates and all duplicates with a coefficient of variation (CV) >10% were reanalyzed. The control samples provided by the manufacturer were within the recommended range. The inter-assay CV based on three different concentrations of control samples was below 9%. Analyses were performed by Ann Burgaz at the Physiology unit, IMM laboratory.

*Paper IV.* To evaluate vitamin D status for study IV, serum 25(OH) D (D<sub>2</sub> and D<sub>3</sub>) was determined in 550 women with LC-MS/MS system consisted of a Shimadzu Prominence HPLC (Shimadzu Corporation) interfaced to a API 4000 triple Quadrupole mass spectrometer (Applied Biosystems) at the Department of Clinical Chemistry, the Malmö University Hospital, Sweden. The reported inter-assay coefficient of variation (CV) for this method were 2.8% at an 25(OH)D concentration of 87 nmol/L and 1.8% at 180 nmol/L.

The quality of both methods was further evaluated using the Vitamin D External Quality Assessment Scheme (DEQAS) and approved. The LC-MS/MS measures were also accredited by Chromsystems, Munich, Germany.

#### 3.2.1.2 Questionnaire

In the examined subgroup an extended 123-item food frequency questionnaire was used to assess dietary intake of vitamin D. Participants also filled out questionnaires regarding lifestyle. Women participating from 2006 (paper I, II and IV) reported their sun exposure habits through a questionnaire established by Ann Burgaz (Figure 9).

#### 3.2.1.3 Blood pressure

In connection with blood collection, supine BPs were recorded by a skilled nurse on the right arm after 10 minutes of rest with a Sphygma manometer. SBPs and DBPs were read to the nearest even figure and the mean of two values was used in the analyses. PP in study IV was defined as the difference between SBP and DBP ( $PP = SBP - DBP$ ) and MAP as the sum of DBP and one third of PP ( $MAP = DBP + 1/3PP$ ).



Figure 9. The sun exposure questionnaire 2006-2009

Serial number: \_\_\_\_\_

## OUTDOORS- and SUN HABITS

### 1. How much time, per week, do you spend outside during the day?

**Oct-April:** ☐ no time ☐ 1/2h ☐ 1h ☐ 2h ☐ 4h ☐ 6h ☐ more

**May-Sep:** ☐ no time ☐ 1/2h ☐ 1h ☐ 2h ☐ 4h ☐ 6h ☐ more

### 2. How often do you sunbathe in a solarium?

☐ Every week ☐ Twice a month ☐ Once a month ☐ A few times a year ☐ Never

When did you last sunbathe in a solarium? \_\_\_\_\_

### 3. How often do you go on a sun vacation?

☐ Once a year ☐ Twice a year ☐ More often ☐ Never

When were you last at a sun vacation? \_\_\_\_\_

### 4. How does your skin get after you being in the sun?

☐ Always burns/never tans ☐ Usually burns/tans with difficulty  
☐ Sometimes mild burn/gradually tans ☐ Rarely burns/tans with ease

### 5. Do you usually use sunscreen when sunbathing?

☐ Yes, always ☐ Sometimes ☐ No, never

### 6. When it is a sunny weather:

☐ Are you preferably in the sun ☐ Both in the sun and in the shadow ☐ Prefer shadow

May we phone you if we have further inquiries? ☐ Yes (Best time to call: \_\_\_\_\_)

☐ No

Name:

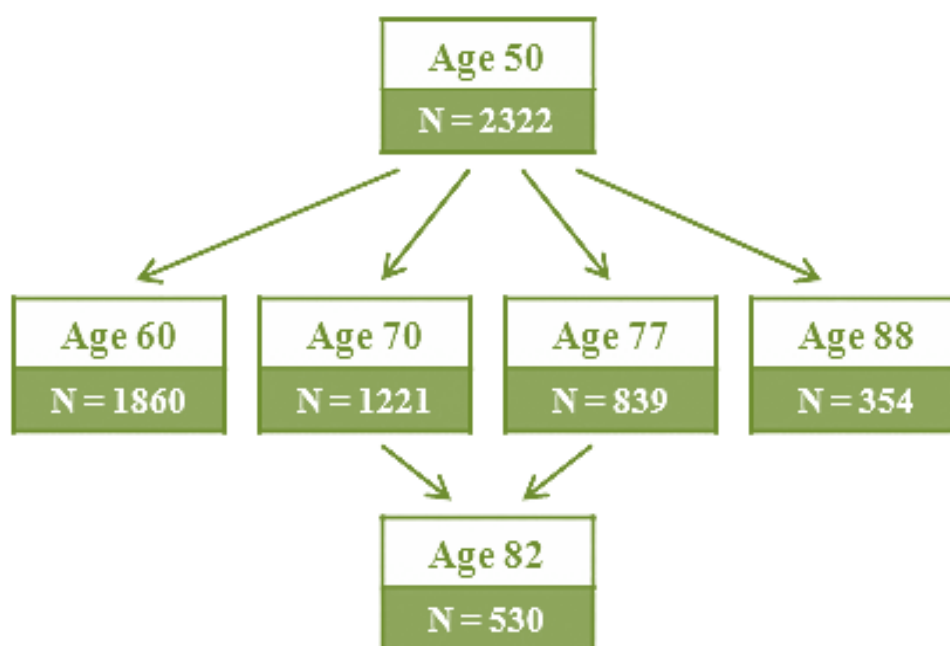
Telephone number:

**THANK YOU FOR YOUR ASSISTANCE**

### 3.3 UPPSALA LONGITUDINAL STUDY OF ADULT MEN (ULSAM)

The Uppsala Longitudinal Study of Adult Men, ULSAM, is a population based cohort study that started in Uppsala, Sweden, in 1970. All men born between 1920-1924 living in Uppsala at that time were invited to participate. Participants were examined at baseline at age 50 and reinvestigated at age 60, 70, 77, 82 and 88 (Figure 10). The six investigations were extensive and carried out in similar manner after an overnight fast. Only data from the age 70 investigation was used in this thesis (paper III). At baseline, 2841 men were invited and 82% (2322) agreed to participate. At age 70, 1681 men were invited and 73% (1221) participated. Men not participating at age 70 did not statistically significantly vary from those who did participate with regard to BMI at age 50. The group who did not participate had a mean BMI of 25.1 compared to 24.8 kg/m<sup>2</sup> for participants (p=0.11).

All participants gave informed consent and the local Ethics Committee of the Medical Faculty at Uppsala University has approved the study.



*Figure 10. A schematic overview of the ULSAM-study. All men born between 1920-24 and living in Uppsala in 1970 were invited to the baseline investigation at age of 50 years. For the age of 60, 70, 77 and 88 years investigation all men alive and still living in Uppsala were invited to participate. For the 82-years investigation those who participated in the 70 and/or the 77 years investigation were invited.*

#### 3.3.1.1 25(OH)D analysis

*Paper III.* Venous blood samples were drawn after an overnight fast and stored at -70° C for  $12.6 \pm 1.1$  year until analysis (Zethelius, *et al.* 2008). 25(OH)D is stable in stored plasma (Hollis 2008). Plasma 25(OH)D concentration was determined with HPLC-atmospheric pressure chemical ionization-MS at Vitas, Oslo, Norway ([www.vitas.no](http://www.vitas.no)). Vitas has a Vitamin D External Quality Assessment Scheme certificate. The inter-assay CV was 7.6% at 47.8 nmol/L and 6.9% at 83.0 nmol/L.

#### 3.3.1.2 Questionnaire, interview and examination

A self-administered questionnaire was used according to Collen and co-workers (Collen, *et al.* 1969) to collect information on various factors including physical activity, medical treatment and previous and current disease. The medical questionnaire was based on the questionnaires previously used for the investigations at age 50 and 60. Leisure time physical activity was assessed using the four questions: 1) Do you spend most of your time reading, watching TV, going to the cinema or engaging in other, mostly sedentary activities?, 2) Do you often go walking or cycling for pleasure?, 3) Do you engage in any active sport or heavy gardening for at least 3 hours every week?, 4) Do you regularly engage in hard physical training or competitive sport? Based on these questions, four physical activity categories were constructed: Sedentary, Moderate, Regular and Athletic. Information on smoking habits (current, former or never smoker) was retrieved through interview reports.

A dietary assessment was performed using a 7-day precoded food record; prepared and validated by the National Food Administration (NFA) (Becker 1998).

Height was measured to the nearest whole cm and weight to the nearest whole kg. BMI was calculated as weight divided by height squared.

#### 3.3.1.3 Office blood pressure

BP was measured twice with a Mercury manometer (Kifa Ercameter, wall-model) and with 2 minutes rest, on the right arm with the subject in the supine position after resting for 10 minutes. SBPs and DBPs were read to the nearest even figure and the mean of two values was used in the analyses. SBPs and DBPs were defined as Korotkoff phases I and V, respectively. Hypertension by office BP in study III was defined as a SBP >140 mmHg and/or DBP >90 mmHg or a regular use of antihypertensive medication. Hypertension treatment was classified as treatment with diuretics, betaadrenergic blocking agents, vasodilator drugs, sympathetic blocking drugs, hydralazines, or combined antihypertensive drugs.

### 3.3.1.4 Ambulatory blood pressure

The twenty-four-hour ambulatory BP was recorded using the Accutracker II equipment (Suntech Medical Instruments Inc, Raleigh NC, USA). The equipment was attached to the participants non-dominant arm by a skilled lab technician, and BP was measured during a 24 hour period starting at 11 a.m. BP recordings were made every 20<sup>th</sup> or 30<sup>th</sup> minute between 6 AM and 11 PM and every 20<sup>th</sup> or 60<sup>th</sup> minute between 11 PM and 6 AM as previously described (Ingelsson, *et al.* 2006). Data were edited by omitting all readings of zero, all heart rate readings <30, DBP readings >170 mmHg, SBP readings >270 and <80 mmHg, and all readings where the difference between SBP and DBP was less than 10 mmHg. Subjects with  $\geq 4/10$  hours of recorded and technically satisfactory BP during daytime, and respectively,  $\geq 3/6$  hours of recorded BP during night-time, were included in the study. SBP and DBP were given by the auscultatory device. The CVs for 24-hour SBP and DBP were 6.8% and 5.5% respectively. Hypertension by ambulatory BP in study III was defined as a SBP >130 mmHg and/or DBP >85 mmHg or a regular use of antihypertensive medication. Hypertension treatment was classified as described above.

### 3.3.1.5 Confirmed hypertension

In Table 2. the four different classifications that were possible to be used with both office and ambulatory BP measurements are shown. In the four groups we have normotensive men at both measurements, white-coat hypertensive office BP and normotensive in 24h BP, masked hypertensive according to office BP and hypertensive in 24h BP and hypertensive in both groups, respectively.

Table 2. Classification of the study subjects in paper III into confirmed normotensive and confirmed hypertensive categories

	Office BP SBP >140 DBP >90	24h BP SBP >130 DBP >85	Confirmed normotensive	Confirmed hypertensive	Frequency
<b>Included</b>	0	0	1	0	<b>184</b>
<b>Excluded</b>	1	0	0	0	102
<b>Excluded</b>	0	1	0	0	76
<b>Included</b>	1	1	0	1	<b>649</b>

### 3.4 STATISTICAL ANALYSIS

The univariate associations were assessed using Pearson's, Spearman's correlation coefficient (r) and Kendal's tau. Kruskal-Wallis or analysis of variance (ANOVA) was used to assess differences between several independent groups and Mann-Whitney to assess differences between two independent groups.

#### 3.4.1 Determinants of vitamin D (paper I and II)

Multiple linear regression (MLR) analysis was used to explain the relationship between the dependent variable, i.e. serum 25(OH)D concentrations, and the explanatory variables. The MLR is represented by the following equation:

$$Y_i = B_0 + B_1x_{i1} + B_2x_{i2} + \dots + B_px_{ip} + E_i \text{ where } i = 1, 2, \dots, n$$

Where:  $B_0$  is the constant term and  $B_1$  to  $B_p$  are the coefficients relating the  $p$  explanatory variables to the variable of interest.

#### 3.4.2 Vitamin D and hypertension (paper III)

Unconditional logistic regression was used to investigate the association between the exposure factor, plasma 25(OH)D concentrations, and the outcome hypertension. Logistic regression analysis was carried out as univariate and multivariate. The variables included in the multivariate logistic regression models were BMI, physical activity, alcohol intake, month of blood sampling, smoking behavior, S-calcium, S-phosphate, S-creatinine and S-uric acid. The results are presented as odds ratios (OR) with 95% confidence intervals (95% CIs). A nonlinearity test was obtained by fitting a restricted cubic spline model with three knots.

#### 3.4.3 Vitamin D and blood pressures (paper IV)

In paper IV quantile regression was used as this approach permits estimating various quantile functions of a conditional distribution, among them the median function is a special case. Each quantile regression exemplifies a particular point of the conditional distribution; putting different quantile regressions together thus provides a more complete description of the underlying conditional distribution. This analysis is useful when the conditional distribution is heterogeneous and does not have a "standard" shape.

### 3.4.4 Confounders

Confounders were selected when associated with both the exposure and the outcome or factors that changed the risk estimate by more than 10% when entering the crude model. Traditional risk factors for vitamin D deficiency, hypertension and blood pressures were included as potential confounding factors. The variables included in the multivariate logistic regression models were age, BMI, smoking status, history of CVD, month of blood collection and physical activity.

All tests were two-sided. Statistical analyses were accomplished by using SPSS, version 14.01 (SPSS Inc., Chicago, IL, USA) in paper I and II, SAS, version 9.2 (SAS institute Inc., Cary, NC, USA) in paper III and IV and Stata, version 10 (Stata Corp, College Station, Texas, USA) in paper IV and V. A p-value of less than 0.05 was regarded as statistically significant.

### 3.4.5 Meta-analysis (paper V)

To summarize the evidence regarding the association of circulating 25(OH)D concentrations and hypertension a meta-analysis of published epidemiologic studies was performed. The data report follows to the guidelines proposed by the Meta-analysis of Observational Studies in Epidemiology (MOOSE) group (Stroup, *et al.* 2000).

#### 3.4.5.1 Search Strategy

Relevant studies were identified by searching in Pub Med and EMBASE databases for articles published until November 2010, and by manually searching the reference lists of relevant publication. To discover studies of interest the following search terms were used to the computerized search: *25(OH)D*, *25-hydroxyvitamin D*, or *vitamin D* combined with *hypertension*, *hypertensive* or *blood pressure*. No language restriction was enforced though the search criteria were restricted to studies of humans.

#### 3.4.5.2 Study Selection

Studies were included if they presented original data from cohort, case control or cross-sectional studies and reported risk estimates such as risk ratios, hazard ratios or odds ratios with 95% confidence intervals, or provided sufficient data to permit their calculation. The exposure was plasma or serum 25(OH)D concentration and the outcome was hypertension. Only studies on adults were included. To avoid violating independence assumptions, studies were included only once. We therefore decided that when there were multiple publications from the same study population, the risk

estimate adjusted for the most appropriate confounding factors comparable to the other studies included in the dose-response analysis were used.

#### 3.4.5.3 Data Extraction

The following data were extracted from each publication: first authors' last name, year of publication, quality score, country of population studies, ethnicity of study population, investigation time period, sex, age, study design, sample size as cases and controls, method of measuring circulating 25(OH)D concentrations, midpoint for highest versus lowest category, BP cut-offs for classifying hypertension, risk estimates with corresponding CIs for circulating 25(OH)D concentrations, and variables controlled for in the analyses that reflected the greatest degree of adjustment for potential confounders.

#### 3.4.5.4 Statistical Analysis

In the meta-analysis (paper V), highest vs. lowest 25(OH)D concentration categories were compared within the specific studies. The summary OR estimates with its 95% confidence interval were derived using the method of DerSimonian and Laird (DerSimonian and Laird 1986) and the assumption of a random effects model, which incorporated between-studies variability.

Dose-response random-effects meta-regression analysis from the correlated natural log of the ORs were conducted across categories of 25(OH)D concentration (Greenland and Longnecker 1992, Orsini 2006). This method requires that the distribution of cases and non-cases or person-time and the OR/RR with its variance estimate for at least three quantitative exposure categories are known. For each study, the median or mean level of circulating 25(OH)D concentration for each category was assigned to each corresponding OR/RR. When the median or mean concentration was not reported, we assigned the midpoint of the upper and lower bound in each category as the average concentration. If the upper bound in the highest category or the bottom in the lowest category was not provided, we assumed that it had the same amplitude as the preceding category.

Statistical heterogeneity among studies was evaluated using the  $Q$  and  $I^2$  statistics (Higgins and Thompson 2002). The  $Q$ -test is a chi-square test with degrees of freedom equal to the number of studies minus one, and is used to test the null hypothesis that the difference between the study estimates of OR/RR is due to chance (the smaller the  $p$  value, the larger is the heterogeneity among studies). The  $I^2$  statistic [ $I^2 = (Q - (k-1)) / Q$ ] describes the percentage of total variation in estimates of the OR/RR

across studies that is due to heterogeneity rather than chance (Higgins and Thompson 2002). Random effects meta-regression analysis was used to examine sources of heterogeneity and to provide an estimate of unexplained heterogeneity (Thompson and Sharp 1999). Subgroup analyses were conducted by ethnicity in the studies.

Publication bias was assessed by Egger's regression asymmetry test (Egger 2001).

The results are presented graphically, whereby squares represent study-specific estimates and diamonds represent summary estimates. The area of each square is proportional to the inverse of the variance of the natural logarithm of the relative risk; 95% CIs for individual studies are represented by horizontal lines and for the summary estimates by the width of the diamond. All statistical analyses were two-sided and performed with Stata, version 10 (Stat Corp, College Station, Texas, USA).



## 4 RESULTS

### 4.1.1 Associations of diet, supplement use, and ultraviolet B radiation exposure with vitamin D status in Swedish women during winter at latitude 60° N (Paper I)

Among women in our study the multiple linear regression model showed that taking a sun vacation was the most effective way to get increased serum 25(OH)D concentrations. Dietary vitamin D intake and regular supplement use also increased serum 25(OH)D concentrations. Regarding dietary sources of vitamin D, intake of fatty fishes and fortified dairy products were the most important factors. The model explained 13% of the variance in serum 25(OH)D concentrations (Table 3).

The 116 women's mean serum concentration of 25(OH)D was 69 nmol/L (range: 35-147 nmol/L). Differences in serum 25(OH)D concentrations were observed in relation to several factors. Women who used dietary supplements regularly had 17% higher concentrations than those who did not, those who went for a sun vacation during the previous 6 month had 16% higher levels than those who stayed in Sweden and those with normal weight had 13% higher levels than overweight women according to BMI; all differences were statistically significant.

*Table 3. Factors predicting the concentration in serum 25(OH)D assessed by multiple linear regression*

Independent variables	Serum 25(OH)D (nmol/L)	
	Unstandardized coefficients <sup>1</sup> (95% confidence interval)	Standardized coefficients <sup>1</sup>
Fatty fish (2-3 servings/week)	10.2 (2.6-18)	0.24
Vitamin D fortified reduced-fat dairy products (300g/day)	6.2 (0.8-12)	0.20
Supplement use (regular)	11.0 (0.1-22)	0.17
Sun vacation during winter <sup>2</sup> (yes)	14.5 (5.3-24)	0.28

<sup>1</sup>Regression coefficient ( $\beta$ ).

<sup>2</sup> Travel to latitude with a high UVB-radiation during the previous six months of wintertime

In a subgroup of 72 women with no supplement use and no recent sun vacation, the correlation between serum 25(OH)D concentrations and dietary vitamin D intake was higher than in the study population (116 women) as a whole. Among those 72 women, every 3.5µg/day (7.5µg/day is the recommended dietary intake for adults) extra in dietary vitamin D intake resulted in a 14.8 nmol/L increase in serum 25(OH)D concentrations. Those who ate fatty fish 2-3 times a week had 26 nmol/L higher serum levels than those who consumed no fatty fish. Women who did not go on sun holiday, compared to those who went on sun holiday, were more often vitamin D deficient according to different deficiency criteria (Dawson-Hughes, *et al.* 2005, Lips 2001) (Figure 11).

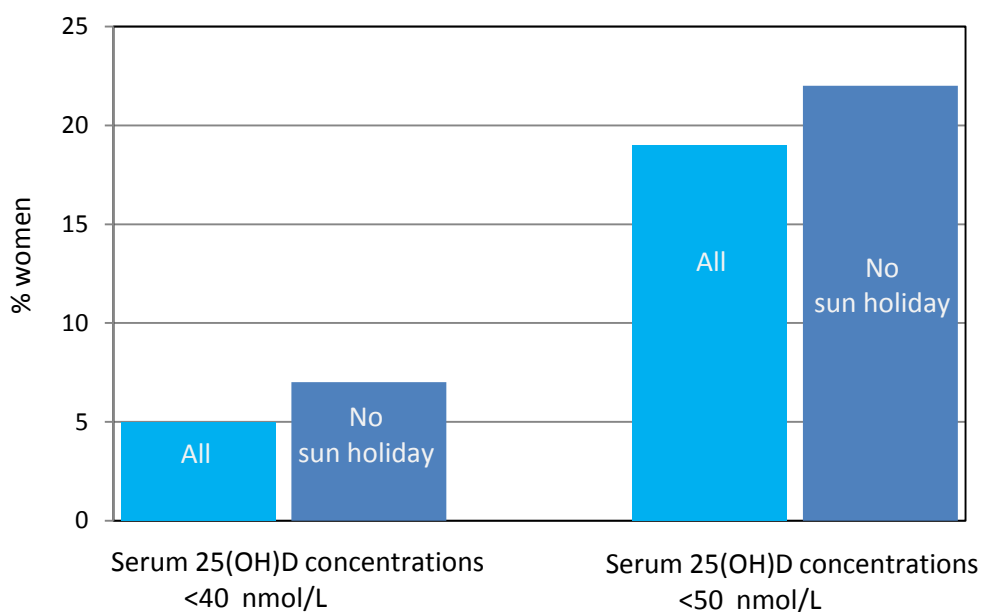


Figure 11. Percentage of women with vitamin D deficiency defined during winter (Jan-March) according to different criteria.

#### 4.1.2 25-hydroxyvitamin D accumulation during summer in elderly women at latitude 60°N (Paper II)

From January to March 2006 we investigated 116 women and during late summer (August-September) 2006 one hundred of them participated again and donated blood for serum 25(OH)D measurement. Among the 100 women the difference between summer- and winter- mean serum 25(OH)D concentrations was 27 nmol/L, statistically significant (Table 4).

*Table 4. Vitamin D status and characteristics of the 100 women (61-83 years old) as assessed during winter (January to March) and summer (August to September) 2006.*

	Winter	Summer
	Mean $\pm$ SD ( range)	Mean $\pm$ SD ( range)
<b>Vitamin D status</b>		
Serum 25(OH)D, nmol/L	72 $\pm$ 23 (36-146)	99 $\pm$ 29 (45-179)
Increase in serum 25(OH)D during summer, nmol/L		27 $\pm$ 21 (-59*-114)
<b>Characteristics</b>		
Dietary vitamin D intake <sup>1</sup> $\mu$ g/day	5.9 $\pm$ 1.9 (3-12)	6.2 $\pm$ 1.7 (3-12)
IU/day	236 $\pm$ 76 (120-480)	248 $\pm$ 68 (120-480)
Regular supplement users <sup>2</sup> %	17	19
Sun vacation during the last 6 months %	27	33

<sup>1</sup> Not including supplements

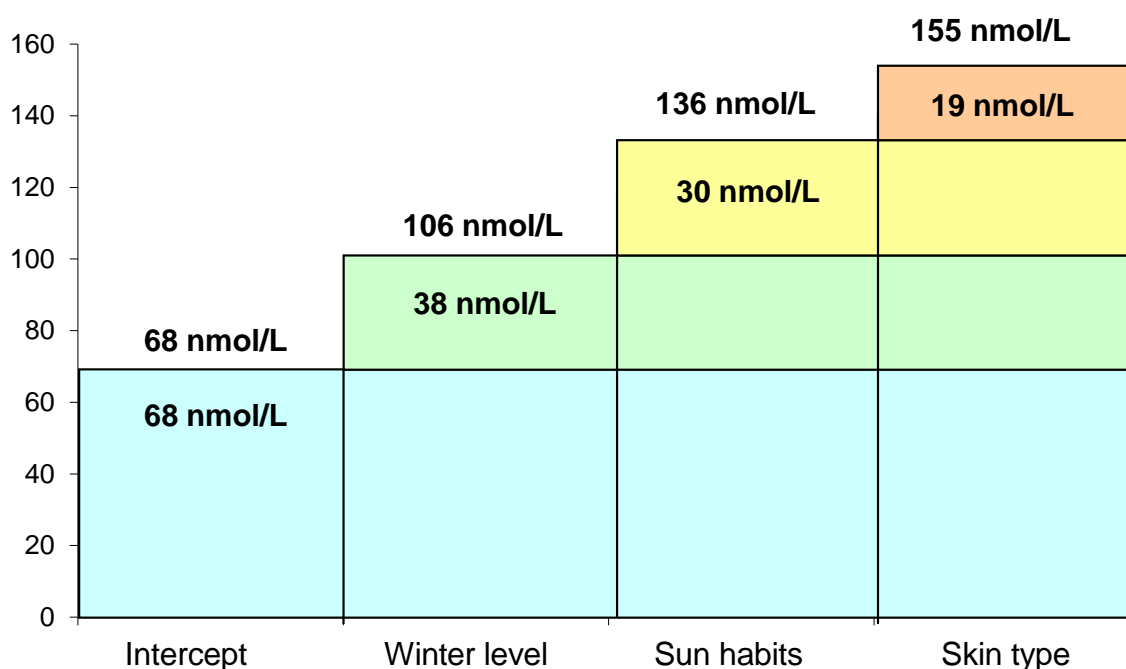
<sup>2</sup> Indicates use of Vitamin D preparations or Multivitamins

\* One participant's serum 25(OH)D concentration was 139 nmol/L during the winter (3 sun vacations) and 80 nmol/L during summer (59 nmol/L decrease)

Serum 25(OH)D concentrations during winter were not influenced by sun habits or skin type in contrast to those measured during the summer. During the late summer sun habits (categorized into preferring to be in the shade, preferring a blend of shade and sun or preferring to be in the sun), skin type (according to Fitzpatrick's scale (Fitzpatrick 1975)) and BMI were correlated with serum 25(OH)D concentrations. However sun vacation, dietary intake and supplement use were not correlated with serum 25(OH)D concentrations during late summer as they were during winter in the

same population. Sun habits were statistically significantly correlated with serum 25(OH)D concentrations during summer ( $r=0.28$ ;  $p=0.004$ ) and those who preferred to be in the sun had 50 nmol/L higher levels than those who preferred the shade. Skin type was positively correlated with summer 25(OH)D concentrations ( $r=0.30$ ;  $p=0.002$ ). Women with the most sensitive skin type had an average serum 25(OH)D concentration of 87 nmol/L while those with less sensitive skin had an average of 103 nmol/L. In addition, skin type was also correlated with sun habits ( $r=0.22$ ;  $p=0.006$ ). Those with most sensitive skin type tend to prefer shade.

The major factors predicting serum 25(OH)D concentrations during late summer were investigated in a multiple linear regression model and were found to explain 50% of the variance in serum 25(OH)D levels. In the model, higher summer serum 25(OH)D concentrations were associated with higher winter 25(OH)D concentrations, a preference to be sun exposed, having non-sensitive skin type and lower BMI (Figure 12). In this model we observed that preferring sun, having a non-sensitive skin type and normal weight was associated with a 64 nmol/L higher serum 25(OH)D concentration during the summer when compared with preferring shade, having a sensitive skin type and being obese.



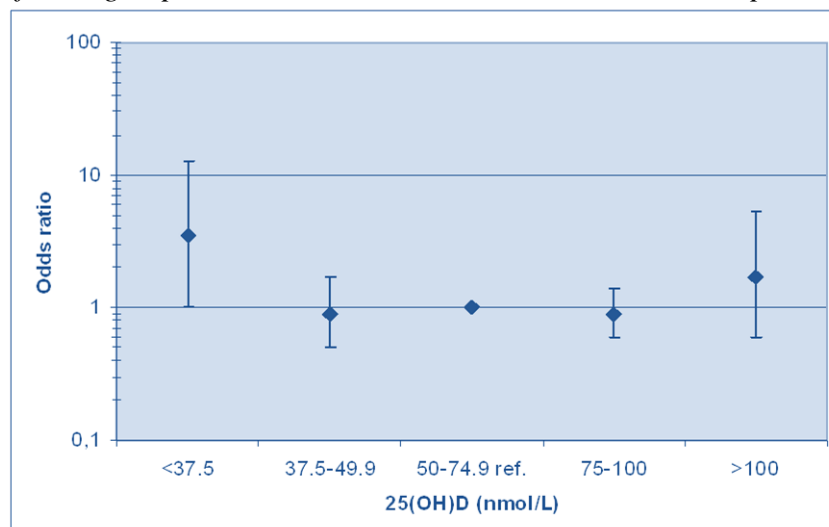
*Figure 12. Major factors predicting concentrations of serum 25(OH)D during summer (August-September) in 100 women (61-83 years old) living at latitude 60° N (estimates are based on a multiple linear regression analysis and added one by one).*

#### 4.1.3 Confirmed hypertension and plasma 25(OH)D concentrations amongst elderly men (Paper III)

The whole study population was investigated when men were ~70 years old. The mean plasma 25(OH)D concentration was  $69 \pm 18$  and on average 3% lower among men with confirmed hypertension compared with the group having normal BP. BMI was the only factor that was statistically significantly different between the confirmed hypertensive and the normotensive group. Alcohol intake was also higher in the confirmed hypertensive group, but not statistically significant.

Men with plasma 25(OH)D concentrations  $<37.5$  nmol/L (4% of the whole study population) had more than a 3-fold increased prevalence of hypertension compared to men with concentrations  $\geq 37.5$  nmol/L in both the univariable and multivariable models. Additional adjustment for S-calcium, S-phosphate, S-creatinine and S-uric acid gave an OR=2.9 (95% CI: 0.9-10.0). Taking smoking into account did not change the prevalence of hypertension. Categorization of plasma 25(OH)D concentrations into five groups revealed an OR for the lowest group ( $<37.5$  nmol/L) of 3.2 (CI 95%:1.0-11.1) compared to the reference group (50-75 nmol/L), which was the biggest group (49% of the whole study population). The group with the highest plasma 25(OH)D concentrations also showed a higher prevalence of hypertension, OR=1.4 although this was not statistically significant (Figure 13). A test for non-linearity was marginally statistically significant ( $p=0.095$ ).

*Figure 13. Risk for confirmed hypertension among elderly men in five plasma 25(OH)D categories. Odds ratios (ORs), plotted on log scale with 95% confidence intervals. Numbers of confirmed hypertensive vs. confirmed normotensive men in each of the 5 groups were: 32/3, 71/20, 319/91, 205/65, 22/5, respectively.*

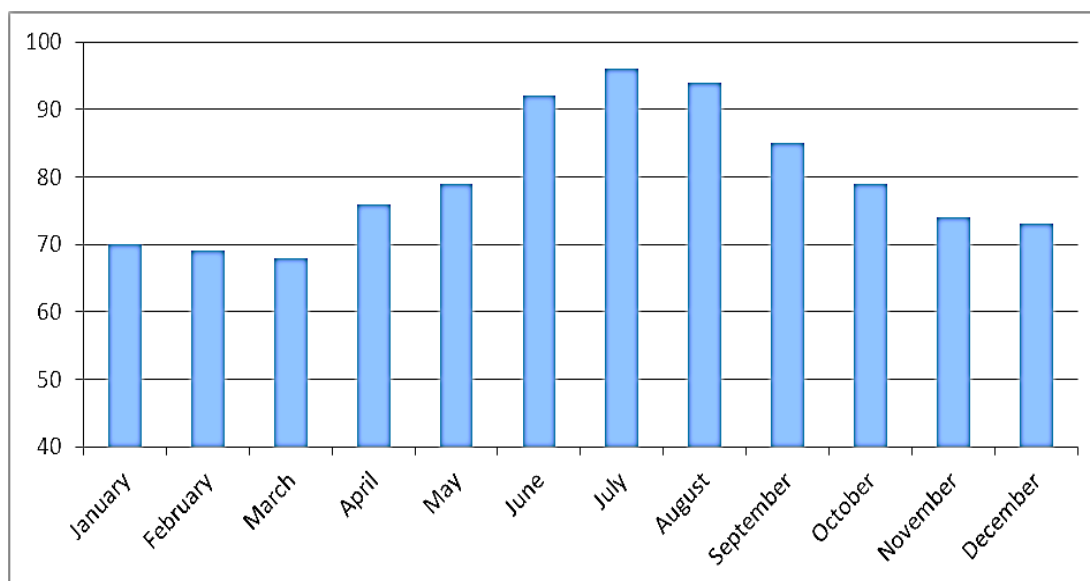


*The model was adjusted for BMI, physical activity, alcohol intake and month when blood sample was collected.*

#### 4.1.4 Serum 25(OH)D concentrations in relation to blood and pulse pressure in elderly women (Paper IV)

The women in this paper had a mean age of 65 years and a mean serum 25(OH)D concentrations of 79 nmol/L. Mean concentrations were lowest during March (mean 68 nmol/L) and highest during July (mean 96 nmol/L) (Figure 14).

*Figure 14. Mean 25(OH)D concentrations by each month for the whole year*



We categorized the participants into four groups; <50 nmol/L, 50-75 nmol/L, 75-100 nmol/L,  $\geq 100$  nmol/L and investigated the association of serum 25(OH)D concentrations with systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP) and mean arterial pressure (MAP) by logistic quantile regression (Table 5). The model was adjusted for age, BMI, smoking status, history of CVD, month of blood collection and physical activity. The observed differences in the 25<sup>th</sup> percentile of PP among women with 25(OH)D concentrations  $\geq 100$  nmol/L was statistically significant lower (7.2 mmHg) than among women with levels <50 nmol/L. No statistically significant differences were found for the 50<sup>th</sup> and 75<sup>th</sup> percentiles of PP. No association was observed between serum 25(OH)D concentrations and SBP, DBP or MAP.

Table 5. Multivariable adjusted differences in the 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> percentiles of PP, SBP, DBP and MAP (mmHg) by categories of serum 25(OH)D concentration among women.

	Vitamin D categories			
	<50	50-74.9	75-99.9	≥100
	nmol/L	nmol/L	nmol/L	nmol/L
	n = 56	n = 188	n = 209	n = 97
mmHg	Mean	Difference vs. ref.		
<b>Pulse pressure</b>				
25 <sup>th</sup> percentile	49 (ref.)	-2.7 (-6.5, 1.1)	-3.1 (-7.0, 0.8)	-7.2 (-11.9, -2.5)
50 <sup>th</sup> percentile	52 (ref.)	2.2 (-1.4, 5.8)	0.2 (-4.5, 4.9)	-2.8 (-7.8, 2.3)
75 <sup>th</sup> percentile	60 (ref.)	0.2 (-5.5, 5.8)	-0.7 (-6.2, 4.9)	-1.2 (-8.0, 5.7)
<b>Systolic blood pressure</b>				
25 <sup>th</sup> percentile	126 (ref.)	-1.6 (-6.7, 3.5)	-2.4 (-6.5, 1.7)	-5.5 (-11.0, 0.1)
50 <sup>th</sup> percentile	131 (ref.)	0.5 (-5.0, 6.0)	-2.1 (-8.1, 3.8)	-5.1 (-12.2, 2.0)
75 <sup>th</sup> percentile	139 (ref.)	3.6 (-3.5, 10.7)	1.8 (-5.1, 8.7)	2.4 (-7.1, 11.8)
<b>Diastolic blood pressure</b>				
25 <sup>th</sup> percentile	73 (ref.)	0.6 (-3.7, 4.8)	2.5 (-1.5, 6.5)	3.2 (-1.3, 7.6)
50 <sup>th</sup> percentile	77 (ref.)	1.9 (-0.8, 4.6)	1.9 (-0.8, 4.5)	2.0 (-2.1, 6.2)
75 <sup>th</sup> percentile	82 (ref.)	2.9 (-0.6, 6.3)	1.7 (-0.6, 7.1)	3.2 (-0.6, 7.1)
<b>Mean arterial pressure</b>				
25 <sup>th</sup> percentile	91 (ref.)	-0.2 (-4.4, 4.1)	0.4 (-3.2, 3.9)	-1.2 (-6.7, 4.3)
50 <sup>th</sup> percentile	96 (ref.)	2,3 (-1.5, 6.1)	0.3 (-0.3, 3.6)	0.4 (-3.9, 4.8)
75 <sup>th</sup> percentile	100 (ref.)	3.2 (-0.8, 7.2)	0.7 (-3.1, 4.4)	2.2 (-1.8, 6.2)

*The model was adjusted for age, BMI, smoking status, history of CVD, season of blood collection and physical activity*

We further investigated the dose-response relation between serum 25(OH)D concentrations and PP by modeling serum 25(OH)D concentration as a continuous variable. In a restricted cubic spline quantile model we found no evidence of non-linearity for the 25<sup>th</sup> (p=0.44), the 50<sup>th</sup> (p=0.50), and 75<sup>th</sup> (p=0.71) percentile of PP. The linear trends between serum 25(OH)D concentrations and the percentiles of PP are presented graphically in Figure 15.

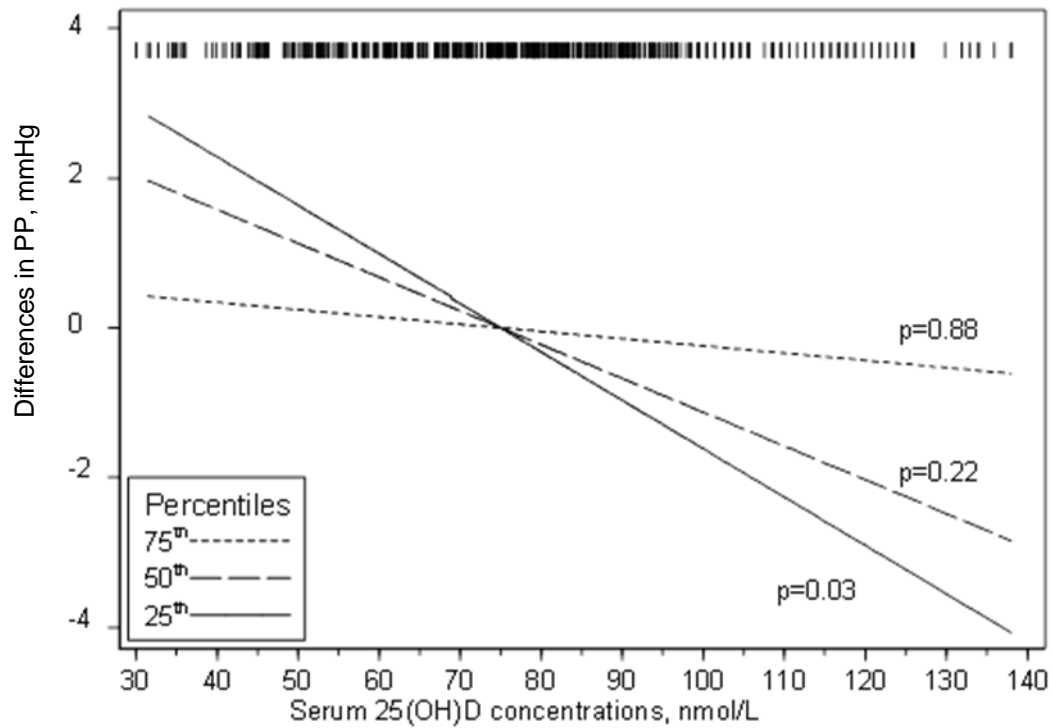


Figure 15. Linear trends of serum 25(OH)D concentrations for predicting the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles of PP estimated with quantile regression and adjusted for age, BMI, smoking status, history of CVD, season of blood collection and physical activity. Tick marks above the fitted trends indicate subjects by their level of serum 25(OH)D concentration.

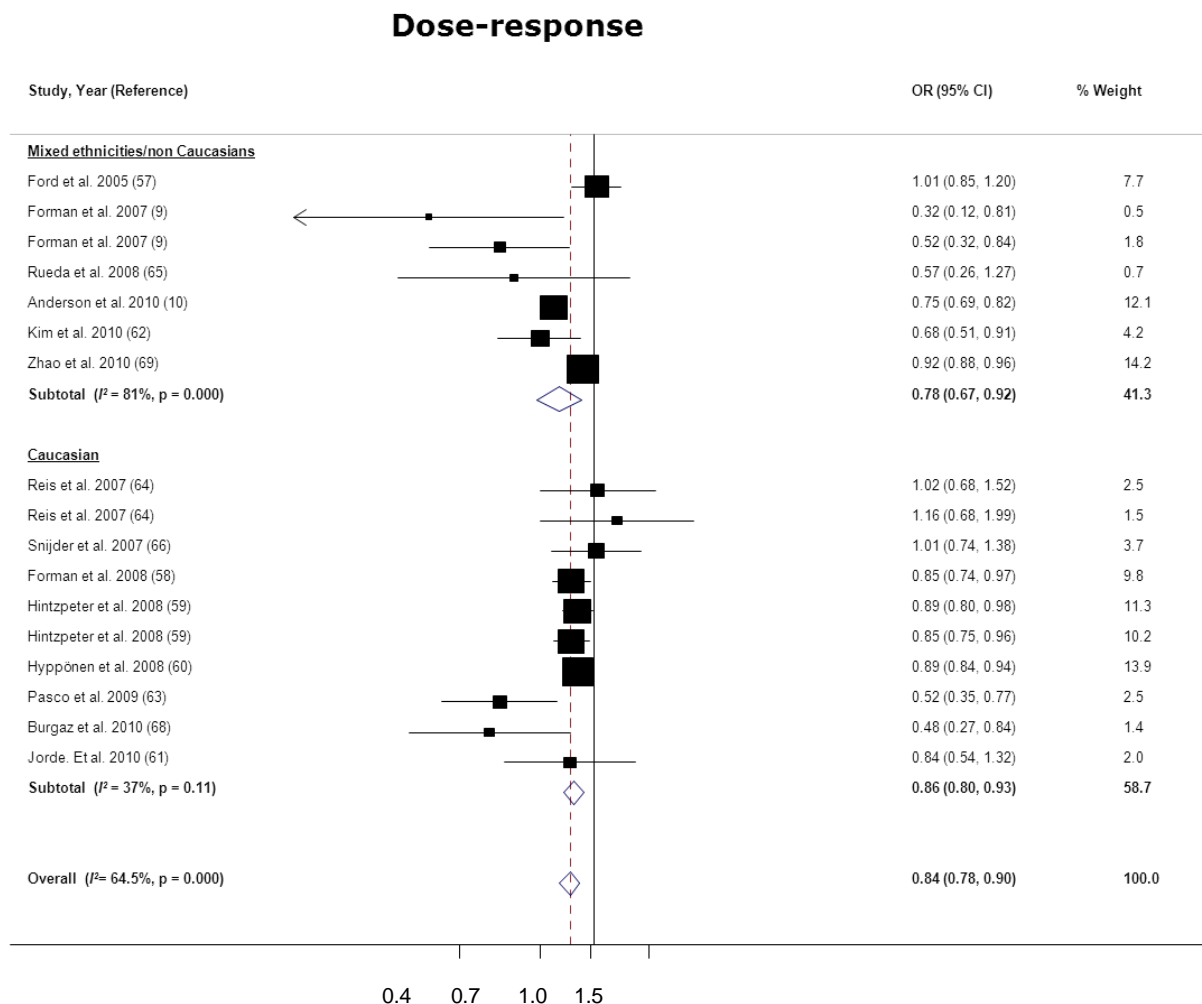


#### **4.1.5 Blood 25-hydroxyvitamin D concentration and hypertension: a meta-analysis (Paper V)**

Three prospective nested case-control studies, one prospective study and 14 cross-sectional studies were included in the meta-analysis (see Table 1. in paper V). Fourteen of the studies reported an inverse association between circulating 25(OH)D concentrations and hypertension (ORs ranging from 0.16 to 0.87) with statistically significant associations being observed in 10 of the studies. Four studies showed a non-significant positive association (OR's ranging from 1.01 to 1.28). Ten studies included only Caucasians, one only Hispanics, one only Asians and five included a mixed ethnicity or nonwhite group. All studies were published between 2005 and 2010 and involved a total of 76 028 participants. In the analysis of all studies, the summary odds ratio of hypertension for individuals in the highest relative to the lowest category of 25(OH)D concentrations was 0.73 (95% CI, 0.63-0.84). The observed heterogeneity among studies of circulating 25(OH)D concentrations and hypertension was explained by difference in ethnicity. Stratifying by only Caucasians versus studies with mixed ethnicities or non-Caucasians indicated no heterogeneity among studies with only Caucasians ( $P=0.25$ ;  $I^2=23\%$ ).

In the multivariable-adjusted estimates for the highest versus the lowest category of blood 25(OH)D concentration the pooled estimate for studies with only Caucasians participants was 0.76 (95% CI, 0.63-0.91) and for the group with mixed ethnicities or non-Caucasians the estimate was 0.69 (95% CI, 0.55-0.86). There was no evidence of publication bias (Egger's test:  $P=0.36$ ). In the dose-response relationship between circulating 25(OH)D concentrations and hypertension, based on 17 studies, the estimate for a 40 nmol/L increment in circulating 25(OH)D concentration was 0.84 (95% CI, 0.78-0.90). In stratified analysis, the estimate was 0.78 (95% CI, 0.67-0.92) among mixed ethnicities or non-Caucasians and 0.86 (95% CI, 0.80-0.93) among Caucasians (Figure 16).

Figure 16. Adjusted odds ratios (OR) of hypertension for dose-response pooled estimates for an 40 nmol/L (16 ng/mL)(corresponding to approximately 2 SDs) increase of blood 25(OH)D concentrations stratified by ethnicity (mixed ethnicities/non Caucasians and Caucasians).



OR indicates odds ratio

CI indicates confidence interval.

The size of each square is proportional to the study's weight (inverse of variance).

## 5 DISCUSSION

### 5.1.1 Main findings and general discussion

#### 5.1.1.1 Determinants of vitamin D status

The pre-hormone vitamin D that humans obtain from dietary intake and sun exposure affects circulating 25(OH)D concentrations. Recommendations vary between different countries for vitamin D intake, vitamin D supplement use or UVB exposure. Also, no agreement exists regarding the optimal circulating 25(OH)D concentrations.

During winter in Sweden (central Sweden 60° N) it is not possible to produce 25(OH)D from sunshine UVB-radiation and therefore the findings from winter can also be applied to populations who have very little or no vitamin D from sun exposure even during summer, e.g. elderly who do not have the possibility to be outside during summer or veiled women who for religious or cultural reasons are completely covered when being outside. During winter, except for sun vacations, the only contributors to vitamin D status were dietary intake and supplement use. In contrast, during summer UVB-related factors but no diet factors influence vitamin D status, which was clearly shown in the study (Paper I). Sun habits, skin type, BMI (overweight people may avoid sun exposure) and preceding winter 25(OH)D concentrations affected the vitamin D status in summer. Sun exposure is such a strong predictor during summer that dietary intake does not emerge as significant determinant of vitamin D during that season.

According to previous studies it seems that groups with the lowest circulating 25(OH)D levels more easily may induce 25(OH)D and therefore can get a larger increase in circulating 25(OH)D concentration after supplementation compared to those with higher 25(OH)D levels (Brannon, *et al.* 2008, IOM). Thus in the discussion regarding the amount of supplementation needed to attain reasonable 25(OH)D levels, this fact should be taken into consideration, because individuals can respond differently depending on their previous vitamin D status (Brannon, *et al.* 2008).

Vitamin D levels from preceding winter were a predictor for summer 25(OH)D levels and also preference for being in sun or shade had a clear influence on the serum 25(OH)D concentrations. Having a non-sensitive skin type with increased skin pigmentation may reduce the capacity to synthesize 25(OH)D. In contrast to other studies in Caucasians (Holick 2008), we observed that a less sensitive skin type predicted higher summer 25(OH)D concentrations than a sensitive skin type. We also observed that those with less sensitive skin type were more willing to be in the sun than in the shade which may explain the results. Persons with sensitive skin get the recommendation to avoid excessive UV radiation to prevent skin cancer (Rigel 2008,

Schwartz and Blot 2006, Tuohimaa, *et al.* 2007). Regarding high BMI as a predictor of lower serum 25(OH)D concentrations, avoidance of UV-radiation can partly be an explanation because obese individuals may be more likely to avoid sun for many reasons and therefore are at additionally higher risk for vitamin D deficiency. (Compston, *et al.* 1981, Wortsman, *et al.* 2000). High BMI as a predictor for lower serum 25(OH)D concentrations observed in our study is in line with earlier studies (Botella-Carretero, *et al.* 2007, Goldner, *et al.* 2008, Vilarrasa, *et al.* 2007).

Our findings indicate that dietary intake is an important predictor for serum 25(OH)D during winter. Even though vitamin D intake among elderly Swedes does not reach the recommended levels (Table 1), circulating 25(OH)D concentrations are among the highest in Europe (Table 6). The finding that, compared to other European countries, Swedes seem to have one of the highest vitamin D intakes (Table 1) can be explained by the use of fortified foods (margarine and low fat dairy products) and a higher consumption of fatty fishes in Sweden. Although Nordic countries are believed to be particularly vulnerable to poor vitamin D status due to low UV radiation during winter it seems that living in sunny climates does not ensure vitamin D sufficiency (Kimlin 2004). Contrary to what is expected, many studies have come to the conclusion that circulating 25(OH)D concentrations are generally higher among people in northern Europe than among people in southern Europe (Lips, *et al.* 2001, van der Wielen, *et al.* 1995). That may not only be explained by a high dietary intake of vitamin D. Vitamin D status in our population is only modestly determined by lifestyle factors such as dietary intake of vitamin D or individual sun exposure and these factors could explain only 13% of the variance in our study population (Paper I). The main determinants of circulating 25(OH)D concentrations at high northern latitudes might be genetic factors (Snellman *et al.* 2009). There are several potential ways in which genetic factors could influence circulating 25(OH)D concentrations such as an individual skin capacity to generate vitamin D, 25(OH)D transport by the vitamin D-binding protein (VDBP) and the hydroxylation of pre-vitamin D to 25(OH)D by the enzyme 25-hydroxylase. Another important aspect, often forgotten in discussions, is the fact that different 25(OH)D assays used in different populations may not give comparable values (Figure 7, page 16). Indeed, the differences in the measured concentrations of a standard reference sample varied up to 65% for 25(OH)D<sub>3</sub>. The even larger differences for 25(OH)D<sub>2</sub> are not of great importance in Sweden/Europe because circulating 25(OH)D<sub>2</sub> contributes only with ~1% of the total circulating 25(OH)D. In USA the percent of circulating 25(OH)D<sub>2</sub> is larger due to fortification and supplement with vitamin D<sub>2</sub>. Therefore all 25(OH)D assays should be validated by an external quality assessment scheme, such as DEQAS, and calibrated, if possible, to get comparable values. The best solution in a future would be to get consensus which method should be used and use only this method.

Table 6. Vitamin D status in middle-aged and adult (non-institutionalized) European populations during the whole year or only winter

Country	Age (sex, n)	Latitude	Mean circulating 25(OH)D (nmol/l)	Analytic method
Sweden				
(Burgaz et al. 2010)	70-74 (M, 833)	55-69°	69	HPLC
(Burgaz et al. 2011)	59-85(F, 550)	55-69°	79	LC-MS/MS
(Melin, et al. 1999)	>80 (M,F, 81)	55-69°	65 w*	DiaSorin RIA
(Rizzoli, et al. 2006)	41-96 (F, 150)	55-69°	87	Nichols ADVANTAGE
Iceland (Sigurdsson, et al. 2000)	70 (F, 308)	63-67°	53 w	DiaSorin RIA
Norway (Tuohimaa, et al. 2004)	<40-60 (M, 258)	58-71°	50 w	DiaSorin RIA
Finland (Lamberg-Allardt, et al. 2001)	31-43 (M,F, 328)	59-69°	46 w	DiaSorin RIA
Estonia (Kull, et al. 2009)	25-70 (M,F, 367)	57-59°	52	DiaSorin RIA
Denmark (Brot, et al. 2001)	45–58 (F, 2016)	54-57°	63	Competitive assay
Ireland (McKenna, et al. 1985)	69 (M,F, 30)	52-55°	21 w	Competitive assay
Netherlands (Lips, et al. 1987)	76 (M,F, 74)	51-54°	33	HPLC
Hungary (Bhattoa, et al. 2004)	41-91 (F, 319)	46-49°	48 w	DiaSorin RIA
United Kingdom (Boonen, et al. 1997)	>65 (F, 800)	50-58°	57	Competitive assay
Belgium (MacFarlane, et al. 2004)	21-65 (M,F, 126)	49-51°	49 w	DiaSorin LIAISON
France (Chapuy, et al. 1996)	35-65 (M,F, 1569)	42-51°	61 w	DiaSorin RIA
Italy (Bettica, et al. 1999)	59 (F, 570)	36-46°	45	DiaSorin RIA

\* w = winter values

#### 5.1.1.2 Vitamin D and blood pressure

An inverse relationship between vitamin D and the renin angiotensin system (RAS) activity suggests that vitamin D may act as an endogenous inhibitor of the RAS. Definitive mechanistic and outcome studies to evaluate the effect of vitamin D supplementation on the RAS and BP have yet to be completed (Vaidya and Forman 2010). Other potential mechanisms could include the effects of vitamin D on the cells of the vessel wall, which include endothelial cells, vascular smooth muscle cells, and macrophages, all of which express the vitamin D receptor (VDR) as well as  $1\alpha$ -hydroxylase (Sugden, *et al.* 2008). Therefore an optimal level of circulating 1,25(OH)D which is regulated by 25(OH)D concentrations, is thought to be crucial for a normal level of BP (Li 2003). Our results are in line with these mechanisms and paper III indicate that men with vitamin D levels of  $<37.5$  nmol/L have a 3-fold increased risk for hypertension compared to men with normal levels (50-75 nmol/L). This association has also been observed in other studies (Anderson, *et al.* 2010, Forman, *et al.* 2008, Wu, *et al.* 2009, Zhao, *et al.* 2010, Forman, *et al.* 2007). The results in paper IV show an inverse association between serum 25(OH)D concentrations and pulse pressure (PP) which can be linked to the effects of vitamin D on the cell of the vessel wall. Vitamin D concentrations are positively related to DBP and inversely to SBP, which are determined by stiffness in the artery. Also our results indicate that serum 25(OH)D concentrations affect PP most significantly where it mirrors the level around the cut-point between healthy and unhealthy PP  $\sim 40$  mmHg. That might be due to that higher PP indicates even stiffer arteries, which might than not be reversible by the 1,25(OH)D effect.

Defining the normal range for 25(OH)D levels has to date been controversial (Barrett and McElduff 2010). Circulating 25(OH)D concentrations higher than 375 nmol/L (150 ng/mL) are considered as toxic (Mawer *et al.* 1985). However, already levels  $>220$  nmol/L (88 ng/mL) seems to be accompanied by hypercalcemia and risk for kidney stones (Vieth 1999). There is a growing awareness that vitamin D sufficiency is required for optimal health. A potential role of vitamin D in a range of diseases indicates that different circulating 25(OH)D concentrations may be optimal for different diseases. Some studies also indicate that very high levels are not optimal (Michaelsson, *et al.* 2010, Nielsen, *et al.* 2010, Tuohimaa, *et al.* 2004). As the suggested U-shaped association between plasma 25(OH)D concentrations and hypertension in the paper III other recent observational epidemiology studies have reported an U-shaped association between circulating 25(OH)D concentrations and tuberculosis (Nielsen, *et al.* 2010), prostate cancer (Tuohimaa, *et al.* 2004), total cancer mortality (Michaelsson, *et al.* 2010) and all-cause mortality in humans (Michaelsson, *et al.* 2010) as well as an U-shaped association with the aging process in mice (Tuohimaa

2009). All these results taken together indicate that there can be an optimal range of vitamin D status but both too low or too high circulating 25(OH)D concentrations may have adverse effects on health. In consequence it is assumed that there is a curvilinear risk for the intake of vitamin D, both too low dietary intake and extremely high intakes (supplements) can be associated with increased risk of negative effects, and optimum intakes are still to be defined (Hayes 2008).

#### 5.1.1.3 Meta-analysis

Meta-analyses of vitamin D supplementation and BP reported weak evidence to support a small effect of vitamin D supplementation on BP (Pittas, *et al.* 2010, Witham, *et al.* 2009). The conclusion that can be drawn from the studies on vitamin D supplementation and BP is limited, because of large differences in doses and duration used in the RCTs. Also, the complexity of vitamin D pharmacology makes the optimal strategy for vitamin D supplementation unclear, as pointed out in a recent paper highlighting these problems (Vaidya and Forman 2010). The endogenous production of 25(OH)D (through exposure to UVB radiation) is a more important predictor of vitamin D status than vitamin D intake (Ovesen, *et al.* 2003). In a small trial, UVB exposure 3 times per week (6 minutes/time) during 6 weeks was associated with a 162% increase in plasma 25(OH)D concentrations (57.6 nmol/L (23 ng/mL) to 151.2 nmol/L (60 ng/mL)) and with a 6 mmHg reduction in both SBP and DBP. In hypertensive patients who received UVA exposure, no significant change in plasma 25(OH)D concentrations or BP occurred (Krause, *et al.* 1998).

As discussed earlier, one concern with vitamin D treatment is that there is no consensus about the optimal circulating 25(OH)D concentration and there is no consensus about the vitamin D supplementation dose that significantly improves health outcomes (Pittas, *et al.* 2010, Witham, *et al.* 2009). In order to reach a conclusion about the optimal level of 25(OH)D concentrations there is a lot of factors that have to be taken into consideration. A variety of factors may influence vitamin D status among different ethnicities, but elucidating the primary risk factors for vitamin D deficiency in different ethnicities will require more research involving for example studies of skin color (genes), body composition (lean and fat mass), age- and, gender-differences, dietary and supplement use patterns and sun-protective behaviors. Large studies, taking all these factors into account, using reliable measurements of circulating 25(OH)D concentrations and reliable diagnoses of different diseases are needed to specify optimal 25(OH)D concentrations.

## 5.1.2 Methodological considerations

### 5.1.2.1 Study design

Papers I-IV are based on cross-sectional study design. A cross-sectional study design is most often used when the purpose of the study is descriptive. The purpose can also be to estimate the prevalence of the outcome of interest, for the population or subgroups within the population at a given time point. Cross-sectional studies gather information about the prevalence of health-related conditions, but they cannot distinguish between newly occurring and long-established conditions, because there is no time aspect involved. The fact that exposure information and outcome are assessed at the same time gives no indication whether exposure occurred before, after or during the onset of the disease outcome. Therefore it is not possible to infer causality. Nevertheless, cross-sectional studies indicate associations that may exist and are therefore useful in the generation of hypotheses for future research. Multiple outcomes can be studied, thus this type of study can be relatively fast and cheap. Nearly all studies of circulating 25(OH)D concentrations and hypertension published so far (paper V) are of cross-sectional design.

It is of great importance that the study population used in a cross-sectional study is representative. Therefore high response rate is required both when studying whole source population or a random sub-sample from the source population. These factors determine how well results from cross-sectional studies can be generalized to the whole target population. The estimates presented in this thesis are based on data from two random sub-cohorts of Uppsala women from the population-based SMC with high response rate (paper I, II and IV) and on the whole community-based cohort of Uppsala men, ULSAM, also with high response rate (paper III). Thus in paper I-IV participants should well represent the entire middle-aged and/or elderly female and male Swedish population.

### 5.1.2.2 Selection Bias

Selection bias may be introduced when there are systematic errors in the procedures used to select a study person from the study population or if there are any factors influencing the participation (Rothman 2008, Rothman *et al.* 1998).

The thesis (except meta-analysis in paper V) using cross-sectional design is based on population- and community-based cohorts with high response rates (SMC 74% and ULSAM 82%). Random sub-cohorts had high (paper I, 97% and paper II, 86%) or relatively high (paper IV, 60%) response rate. Thus the associations between the exposures and outcomes should be very much the same among the participants as among non-participants, who could theoretically be eligible for the studies. However,



selection bias may have occurred if the oldest invited women, who may have limited mobility, participated less frequently. Nevertheless, there was no significant difference in, for example, mean BMI (which is related to both vitamin D status and blood pressure) between women participating in study I and II and the whole SMC. Women in study IV had lower mean BMI than the whole cohort. Those women may have a greater interest in their health. Regarding men in the ULSAM study (paper III) there was no statistically significant difference regarding BMI between participants and those who did not participate.

#### *5.1.2.3 Random and systematic errors*

There is always the possibility of random error in quantitative research. Precision is defined as the lack of random error. Precision in a study depends mostly on the sample size, but also on the classification quality of exposure and outcome (Rothman 2008). To provide information about the precision of the association estimates 95% confidence intervals are mostly used. This means that if the data collection and analysis could be replicated many times, the confidence interval should include the correct value of the measure 95% of the time (Rothman 2002).

Systematic error is usually referred to as bias. It does not depend on study size or chance, instead it is a methodological error that is introduced when selecting study participants (as discussed above), defining or assessing exposure or the outcome under study. An estimate that has little systematic error may be described as valid.

Both errors can be divided into categories such as: Information bias, selection bias and confounding (Rothman 2002).

##### 5.1.2.3.1 Information Bias

Information bias arises when measurements and classifications of exposure or outcome are not valid (Rothman 2008). A participant could thus be placed in an incorrect category regarding exposure or outcome. Such misclassification can be divided into differential and non-differential misclassification. Differential misclassification occurs when the misclassification of exposure is different among those with and without the outcome. Likewise, it is also present when misclassification of outcome is different among those with and without the exposure. Differential misclassification can either exaggerate or underestimate an effect. In non-differential misclassification, the misclassification does not differ between exposed or unexposed or those with or without the outcome. This bias may lead to a diluted effect estimate (Rothman 2002).

### *Misclassification of exposure*

#### Paper I and II

Self-reported answers in the questionnaire about consumption of vitamin D containing foods or other factors influencing vitamin D status may introduce misclassification to these exposures. In the random subsample from the SMC, used in paper I and III, any measurement error in the self-reported exposures is unlikely to be differential, i.e. related to the outcome (measured 25(OH)D serum concentrations). However, we cannot exclude non-differential misclassifications, due to random errors in self-reports, which most likely lead to an underestimation of the observed associations in the two studies.

#### Paper III and IV

In both studies the exposures were measured by laboratory assays. Circulating 25(OH)D concentrations were analyzed by LC-MS/MS (SMC) and by HPLC (ULSAM), which are considered the most reliable methods of assessing vitamin D status (Figure 7, page 16). The DEQAS (described at page 16) quality control of the laboratories, where our 25(OH)D analyses were performed, indicated a good performance.

#### Paper V

Our meta-analysis was based on a quantitative summary of results from several studies using different methods for analyzing circulating 25(OH)D concentrations. However, all the laboratory assays used were satisfying and resulting in one point in the Quality score based on Newcastle–Ottawa Scale system (Wells GA *et al.* 2011). Moreover, the dose-response method used in our meta-analysis eliminates some of the problems with comparability between different 25(OH)D assays used in the included studies.

### *Misclassification of outcome*

#### Paper I and II

The quality of the laboratory assay used for measurement of serum 25(OH)D concentrations is of the highest importance regarding quality of the outcome assessment in these studies. Information on outcome, i.e. serum 25(OH)D concentrations, came from enzyme immunoassay (IDS OCTEIA) analyses. To ascertain analytic quality, we analyzed all standards, controls, and samples in duplicate and reanalyzed all duplicates with a CV >10%. Those analyses were also quality controlled by DEQAS (described at page 16) and have shown satisfying results.

#### Paper III and IV

In paper III, the categorization of confirmed hypertensive and confirmed normotensive men by both office BP measurements and by 24h BP measurements, contributes to the high trustworthiness of the outcome quality. In paper IV, well-trained nurses and the use of a mean level of two measurements of office blood pressure for each participant contributed to an increased reliability of blood pressure classification. Furthermore, both in SMC and ULSAM the circulating 25(OH)D concentrations (exposure) were not analyzed until after the original data collection was finished and thus could not have influenced the measurement of BP (outcome). Therefore, systematic misclassification of the disease (hypertension) appears unlikely but some degree of random misclassification cannot be excluded.

#### Paper V

The meta-analysis includes studies with different methods for assessing BP also self-reported hypertension is used. This varying quality of BP assessment resulted in different scores in the Newcastle-Ottawa Scale system used in the investigation (Wells, *et al.* 2011). However, the summary risk estimates did not differ when studies with lower scores were excluded.

#### 5.1.2.4 Confounding

Confounding can be explained as a mixing of effects and may result in both an over- and underestimation of the odds ratio. Confounding is an important issue when discussing causality in epidemiological studies. Confounders are factors that are associated with the exposure and are themselves risk factors for the disease as opposed to being intermediate steps in the pathway from exposure to disease. When potential confounders are unevenly distributed in the exposed and non-exposed groups, the odds ratio may be affected (Rothman 2002). In papers III and IV potential confounders were selected when they were associated with both the exposure and the outcome and/or changed the risk estimate by 10% or more when included in the model. In paper III when additional potential confounders were added to the model, such clinical measurements as S-calcium, S-phosphate, S-creatinine and S-uric acid, it only changed the estimates marginally. Confounding is one of the most important threats to the validity of results from observational studies. In a study of circulating 25(OH)D concentrations and BP, confounding may distort a true relationship, introducing a false association. Individuals who attempt to be healthy are likely to follow a lifestyle that in general includes high intakes of fatty fish, being physically active, maintaining a low body weight, healthy drinking and no-smoking habits. While we attempted to control for potential confounders in the analyses, those confounding factors can be measured with error, and hence residual confounding may remain. Unknown potential

confounders which were not controlled for in the analysis could hypothetically influence the ability to detect an association or the strength of the observed association.

#### 5.1.2.5 *Generalizability*

Generalizability refers to results and findings that are applicable to other individuals than those in the sample studied. Participants of the SMC and ULSAM are from the general random population of central Sweden. The participation rates in both cohorts were high at baseline and the results are most directly generalizable to all the elderly Swedish female and male Caucasian populations. There seems to be different genetic conditions that determine how to absorb and metabolize vitamin D, which may make our findings not directly applicable to other ethnic groups who have potentially different genetic susceptibility. Otherwise, our results are probably generalizable to most urban settings and in most countries at high latitudes (Uppsala city 60° N).

#### 5.1.2.6 *Publication Bias (Meta-analysis)*

Publication bias might be a problem when conducting meta-analyses. The bias is caused by the tendency of researchers to write and submit, and tendency of peer-reviewers and editors to accept and publish results depending on the magnitude of the association. For example, studies observing statistically significant results and published in English are more likely to be published than “negative” studies (showing no association) in other languages. Therefore, studies showing an association and written in English are more probable to be included in a meta-analysis than “negative” studies in other languages that show no association, something that may introduce bias in the summary risk estimates.

In our meta-analyses of circulating 25(OH)D concentrations and hypertension formal statistical tests of publication bias and visual inspections were done. The Eggers regression asymmetry test was used to assess publication bias (Egger, *et al.* 1997). To minimize English-language bias, we included studies published in any language in our search criteria. We did not observe any indication of publication bias in our meta-analysis (paper V).

## 6 CONCLUSIONS

Based on the papers results included in this thesis, in combination with the available scientific literature in the areas covered by these papers, the following conclusions can be drawn:

- Dietary source of vitamin D such as fatty fishes and vitamin D fortified dairy products, are important to the vitamin D status of middle-aged and elderly Swedish women during winter. Supplement use and traveling to southern countries with sun exposure can also improve serum 25(OH)D concentrations when there is lacking UVB radiation during October to April at 60° N.
- Sun exposure habits during the summer are important determinants for serum 25(OH)D concentrations. Dietary intake, vitamin D fortification and supplement, that influence serum 25(OH)D concentrations during winter may not be of importance during summer. Vitamin D status during summer is influenced by the “start” levels of serum 25(OH)D levels during the preceding winter and by sun exposure habits, skin type and BMI.
- Plasma 25(OH)D concentrations, lower than 37.5 nmol/L, in elderly Swedish men are associated with a threefold higher risk of confirmed hypertension compared to the group with levels between 50 and 75 nmol/L. Also the group with plasma 25(OH)D concentrations higher than 100 nmol/L seemed to have a higher risk of confirmed hypertension, although not statistically significant.
- Levels around a “normal” pulse pressure (PP) (40 mmHg) are inversely affected by serum 25(OH)D concentrations in a middle aged and elderly Swedish female population. Consequently, women in the 25<sup>th</sup> percentile of PP (~46.5 mmHg) seem to be most affected by serum 25(OH)D concentrations compared to women with higher PP. There was no statistically significant association between SBP, DBP and MAP and serum 25(OH)D concentrations.
- Circulating 25(OH)D concentration is inversely associated with hypertension. Quantitative summary of the accumulated evidence based on a dose-response meta-analysis of published studies shows that for every 40 nmol/L (16 ng/mL) increase in circulating 25(OH)D the prevalence of hypertension decreased by 16%.

## **6.1 FUTURE RESEARCH**

### **In a broader perspective of an optimal vitamin D status:**

Investigations regarding how UVB exposure, dietary intake, vitamin D fortified food and vitamin D supplements affect circulating 25(OH)D concentrations are needed to establish recommendations for vitamin D intake. In addition, population characteristics such as age, gender, BMI and ethnicity should be taken into consideration.

Another important issue is whether it is possible to optimize UVB exposure, taking into account skin type, ethnicity, latitude and altitude to obtain a sufficient vitamin D status without increasing the risk of skin cancer. Potentially also artificial well defined UVB radiation may be used without risk for skin cancer to increase circulating 25(OH)D concentrations in the future.

To address the above issues it is important to use comparable laboratory methods for assessment of vitamin D status. The best solution in a future would be to get consensus which method should be used and use only this method.

Investigation into how genes are involved in the absorption and metabolism of dietary intake, vitamin D fortified foods, vitamin D supplement and UVB exposure and the effect on circulating 25(OH)D concentrations. In addition, identification of genes that are involved in regulation of circulating 25(OH)D levels that are optimal for good health. There might be important differences in different populations.

### **More specific for blood pressure:**

Both longitudinal and interventional studies, with satisfactory measurements of both circulating 25(OH)D concentrations and BP, are needed to define the optimum vitamin D status. Moreover, groups with different ethnicity should be investigated separately.

In addition, investigations into the association between circulating 25(OH)D concentrations and pulse pressure are needed, in order to establish vitamin D as a predictor of arterial stiffening and vascular ageing.

## 6.2 SAMMANFATTNING (SUMMARY IN SWEDISH)

Funktionen för cirkulerande 25-hydroxyvitamin D (25(OH)D) är framför allt att upprätthålla serum-nivåerna av kalcium inom ett spann som gynnar skelettet. På senare tid kan man lägga till nya indikationer för att vitamin D har fördelaktiga effekter även på andra viktiga funktioner i vävnader som inte primärt är relaterade till mineralmetabolismen. Cirkulerande 25(OH)D-nivåer påverkas både av kosten och ultraviolett B-strålning (UVB) från solen. Många faktorer påverkar möjligheten att höja de cirkulerande 25(OH)D nivåerna med hjälp av UVB-strålningen såsom latitud, tid tillbringad utomhus, hudtyp, ålder, solskydd, kläddvanor mm. Syftet med den här avhandlingen var att studera vilka faktorer som påverkade de cirkulerande 25(OH)D-nivåerna under vinter- respektive sommarsäsongen hos en grupp kvinnor som deltar i den Svenska Mammografikohorten (SMC). Vidare studerades också plasma 25(OH)D-nivåer i förhållande till högt blodtryck hos män från Uppsala Longitudinal Study of Adult Men (ULSAM). Blodtryck, pulstryck och medelartärtryck i förhållande till serum 25(OH)D koncentrationer studerades hos kvinnor från SMC. För att sammanställa resultat gällande cirkulerande 25(OH)D-koncentrationer och högt blodtryck gjordes en metaanalys.

### Studie I-II

SMC startade 1987 då 90 303 kvinnor i Uppsala och Västmanlands län bjöds in att fylla i en kostenkät, vilket 74% av kvinnorna gjorde. Studien följdes upp med nya kost- och livsstilsenkäter 1997 samt 2008-2009. En grupp på 5 022 kvinnor från SMC lämnade blod-, urin- och fettprover samt DXA- och blodtryck-mättes under åren 2003 till 2009. Bland dessa deltagare undersöktes 116 slumpvis utvalda kvinnor i åldrarna 61-86 år beträffande vilka faktorer som påverkade deras serum 25(OH)D-nivåer under vintern. Resultatet av en multipel linjär regression visade att intag av vitamin D-rika livsmedel såsom tex. lax, sill, strömming, makrill och vitamin D-berikade mjölkprodukter var positiva för deltagarnas D-vitamin status. Även att resa på solsemester och/eller att använda kosttillskott innehållande vitamin D gynnade serum 25(OH)D-nivåerna. Av de 116 kvinnor som deltog på vintern undersöktes 100 av dem igen, denna gång på sensommaren. Medelvärde för serum 25(OH)D-koncentrationer hos de 100 kvinnorna var på vintern 72 nmol/L och på sommaren 99 nmol/L. Till skillnad från vinterresultaten, påverkades vitamin D-nivåerna under sommaren, inte av kosten. Däremot påverkades de av vilka serum 25(OH)D-nivåer de haft vintern innan, deras solvanor, BMI och hudtyp.

### Studie III

ULSAM startade 1970 då alla män boende i Uppsala, födda 1920 till 1924, bjöds in att delta i studien. Det var 3 222 män (82%) som deltog och de blev åter inbjudna när de fyllde 60, 70, 77, 82 och 88 år. I den här tvärsnittsstudien undersöktes sambandet

mellan plasma 25(OH)D-koncentrationer och högt blodtryck hos 833 män i 70-års åldern. En logistisk regressionsmodell justerad för konfounders visade att män med plasma 25(OH)D-nivåer lägre än 37.5 nmol/L hade tre gånger så hög förekomst av högt blodtryck än män som hade nivåer över 37.5 nmol/L. En icke statistiskt signifikant 40% ökning av högt blodtryck visades också hos gruppen män med plasma 25(OH)D-nivåer över 100 nmol/L.

#### **Studie IV**

I gruppen från SMC, som lämnat prover samt blodtryck-mätts, undersöktes 550 kvinnor i åldrarna 59-85 år. Resultatet från en "logistisk quantile regressions" modell justerad för konfounders visade att kvinnorna i den lägsta percentilen gällande pulstryck, med serum 25(OH)D-nivåer under 50 nmol/L hade statistiskt signifikant 7,2 mmHg högre pulstryck än de med serum 25(OH)D-nivåer högre än 100 nmol/L i samma percentil. Inga statistiskt signifikanta samband visades när det gällde serum 25(OH)D-koncentrationer och systoliskt blodtryck, diastoliskt blodtryck eller medelartärtrycket hos deltagarna.

#### **Studie V**

För att sammanställa resultaten från alla studier tillgängliga i Pub Med och EMBASE gällande cirkulerande 25(OH)D-koncentrationer och högt blodtryck gjordes en meta-analys. Resultat från en prospektiv studie, tre prospektiva "nested" fall-kontroll studier och 14 tvärsnittsstudier med totalt 76 028 deltagare summerades i en "random-effects model" och en "dos-response random effects meta-regression model". Odds kvoten för förekomsten av högt blodtryck i den högsta jämfört med lägsta nivån av cirkulerande 25(OH)D var 0,73 (0,63-0,84). För 40 nmol/Ls ökning av cirkulerande 25(OH)D-nivåer minskade oddskvoten med statistiskt signifikant 16% för benägenheten att ha högt blodtryck.

#### **Sammanfattning**

Vintertid påverkade vitamin D-intag via kosten, solsemester och intag av vitamin D-tillskott serum 25(OH)D-nivåerna hos kvinnorna från SMC. Sommartid påverkades nivåerna av vilka vinternivåer de haft, solvanor, BMI och hudtyp. Plasma 25(OH)D-koncentrationer lägre än 37,5 nmol/L hos män i ULSAM ökar förekomsten av högt blodtryck mer än 300% jämfört med de som har nivåer högre än 37,5 nmol/L. När det gäller kvinnor i SMC hade gruppen med serum 25(OH)D-nivåer under 50 nmol/L, i den lägsta percentilen, ett pulstryck som var 7,2 mmHg högre än gruppen med serum 25(OH)D-nivåer över 100 nmol/L i samma percentil. En sammanfattning av studier gällande cirkulerande 25(OH)D-koncentrationer och högt blodtryck visade 27% lägre statistiskt signifikanta risk för högt blodtryck vid låga cirkulerande 25(OH)D-nivåer jämfört med höga.



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